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001

Reinforcing dendritic cells for cancer immunotherapy: diverse ways and means to target antigens to human skin

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Dendritic cells (DC) are essential for the induction of primary immune responses, and hence preferred targets for immunization against cancer. Skin DC express C-type lectin receptors such as Langerin or DEC-205 for recognition of (pathogen-derived) antigens. *In situ*, Langerin is expressed mainly on Langerhans cells (LC), whereas DEC-205 is expressed by dermal DC and LC. We aim to load skin-resident DC (i) with antibody-antigen fusion proteins directed against these C-type lectin receptors or (ii) with antigens encapsulated in liposomes coated with a Langerin ligand. (i) Monoclonal antibodies (mAb) were injected intradermally into human skin explants for targeting of skin DC subtypes corresponding to their C-type lectin receptor expression. Langerin mAb was detected exclusively in LC, whereas DEC-205 mAb targeted both dermal DC and LC. A model antigen (EBNA1) fused to DEC-205 mAb elicited EBNA1-specific T cell responses. (ii) Liposomes coated with a Langerin ligand showed exclusive binding to LC in cell suspensions obtained from healthy human skin. These liposomes were rapidly incorporated into LC as visualized by confocal microscopy. Furthermore, to test our vaccination approaches in an *in vitro* model, we generated monocyte-derived Langerhans-like cells, which displayed between 50-80% of Langerin expression on the surface and also showed upregulation of CD83 and HLA-DR upon stimulation with a maturation cocktail. In summary, our study will provide a deeper insight into DC-targeted cancer vaccines, their uptake, intracellular trafficking and antigen processing in skin DC and, hence, resulting T cell responses. Furthermore, liposomes provide a flexible platform that will allow us to encapsulate antigens to investigate their potential for targeted delivery. Those antigen:anti-DC antibody constructs or LC-specific liposomes loaded with antigens will allow to boost pre-existing immunity in patients. Ultimately, this DC-based immunotherapy can be used to increase response rates when used in combination with immune checkpoint inhibitors.

002

A versatile, high-throughput HLA peptidomics pipeline for cancer neoepitope discovery

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Personalized epitope-based vaccines are currently being discussed intensively for tumor immunotherapy. In order to identify epitopes - short, immunogenic peptides (IPs) - suitable to elicit an immune response, IPs of cancer and benign tissue samples are purified using HLA immunoaffinity purification, subsequently analyzed by high performance liquid chromatography coupled to mass spectrometry and finally sequenced performing massively-parallel sequencing. Peptides observed in the HLA ligandome provide direct evidence for the existence of HLA-binding epitopes suitable for

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vaccination.
Computational approaches for processing MS raw data have not been investigated in depth with focus on HLA epitopes. In fact, outcomes of the Human Immunopeptidome Project (HIPP) discussions have pointed out the lack of tailored MS data processing strategies as a major challenge to improve current analysis protocols. Moreover, there is a demand for automated, high-throughput pipelines directly linking raw experimental data available in the PRIDE database to large scale, shared, online repositories such as the SysteMHC Atlas. Several approaches using peptide database search engines have been published. However, as opposed to standard proteomic analysis, no comparisons of the individual search engines and pre- and post-processing tools have been reported.
Here we provide a novel, automated pipeline for HLA ligandomic raw data sets implemented using the OpenMS framework for computational mass spectrometry. Identification, post-scoring and quantification are performed using the recently developed OpenMS 2.2. adapters to Comet, Percolator and the internal tool FeatureFinderIdentification. In contrast to other MS analysis tools, the workflow is guided by MS2 identifications to align MS1 maps and detect suitable peptide features in close proximity, resulting in 99% quantification of all identifications. The availability through KNIME and qPortal allow to combine and customize ligandomics raw data processing in a practical and simple way with a wealth of other immunomics software tools, compatible with any computer operation system or MS instrument.
In a concise benchmark we find that the search engine Comet performs superior over MS-GF+, SequestHT and MaxQuant with regard to the number of peptide identifications. The additional identified peptides are in accordance with known length distributions and sequence motifs without any evidence for a bias. Binding predictions based on allotypic sequence motifs estimate a higher number of HLA binders among the Comet identifications compared to the other search engine findings. Ultimately, we get evidence for a number of neoepitopes that were overlooked in the original publication of public available ligandomics datasets.
We provide the workflow to the science community, with the intention to improve the detection of neoepitopes for successful personalized vaccine therapies.

003

Targeted LC-MS detection identifies novel immunogenic HLA-A2-restricted T cell epitopes derived from HPV16 E6 and E7

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Rational design of a therapeutic HPV vaccine requires knowledge of the epitope repertoire presented on the surface of HPV-transformed cells. However, direct detection of viral or mutation-derived epitopes remains a challenge. The low abundance of these target epitopes and viral immune evasion strategies hinder detection. Furthermore, not every human leukocyte antigen (HLA) class I presented peptide elicits the desired cytolytic immune response. In this study, we investigated HLA-A2 restricted epitopes derived from human papillomavirus (HPV) oncoproteins E6 and E7, as these proteins are attractive therapeutic vaccination targets expressed in all HPV-transformed cells. Our strategy for epitope detection includes preselection of target antigen-derived peptides by *in silico* HLA-binding predictions and *in vitro* binding validation. This is followed by immunoprecipitation of HLA-peptide complexes from HPV-transformed cell lines and elution of bound peptides. Subsequently, our targeted LC-MS^3 approach resulted in successful detection of the previously described HLA-A2-restricted epitope $E7_{11-19}$ and 10 additional E6/E7-derived epitopes. Interferon-γ memory responses against all of these 11 detected peptides could be detected in the blood of healthy donors by ELISpot assays. Further characterization of these responses by a flow cytometry-based cytotoxicity assay showed that $CD8^+$ T cells mediated epitope-specific target cell lysis. Thus, our highly sensitive LC-MS^3 approach is

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suitable for the detection of even low-abundant peptides, a good indicator for immunogenicity and a promising strategy to identify true immunotherapy targets.

004

Predicting observed patient responses to a short-peptide cancer vaccine via clinical trial simulations

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I will present a simulation of the clinical trial, IMA901, a cancer vaccination study in which 9 short peptides highly expressed by renal cell carcinomas (RCC) were injected into the dermis of patients with RCC. In the real trial, it was found that most patients produced T-cell responses to only one or none of these peptides, and that no patient responded to more than three. Considering the differences between patients, we hypothesise that these results are determined by the amount of administered peptide delivered by dendritic cells (DCs) to the draining lymph node, the number of antigen-specific $CD8^+$ T-cells in the lymph node, and the timescales involved.
We developed a mechanistic model of T-cell activation in the lymph node that quantifies the trade-off between these variables, and found that the critical variable that determines immune activation following short-peptide vaccination is the dissociation rate of pMHC complexes. Clinical trial simulations based on this model successfully capture the range of observed IMA901 patient outcomes. The biological and clinical insights derived from these simulations can be used to suggest alterations to the clinical trial design that may have yielded enhanced patient response.

005

The Glioma Actively Personalized Vaccine Consortium (GAPVAC) presents the successful clinical translation of mutated peptide vaccination

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Personalized medicine today frequently means biomarker-guided stratified use of a conventional drug. The Glioma Actively Personalized Vaccine Consortium (GAPVAC) took personalization to the next level by tailoring unique peptide cancer vaccines to each individual patient. The Consortium implemented an immunotherapy, for which the selection of two actively personalized peptide vaccines (APVAC 1 and 2) were used for treatment of patients with newly diagnosed glioblastoma. The two different approaches based on whole-exome sequencing and HLA-ligandome analyses providing information of the actual presentation of relevant epitopes in the tumor. Here we focus on the mutational landscape, composition and biological effects of neo-epitope containing APVAC2 vaccines, clinical data and data for APVAC1 will be presented separately.
GAPVAC-101 (NCT02149225) enrolled 16 patients in a multicenter phase I feasibility, safety and immunogenicity trial integrated into the standard of care. For APVAC1, up to 7 peptides were selected from a warehouse based on individual HLA ligandome, transkriptome and immunogenicity measurements. Peptides containing tumor-specific mutations and non-mutated, individually over-presented peptides not contained in the warehouse have been produced *de novo* for every patient for APVAC2. Preclinical research was conducted to establish the clinical workflow from sample acquisition to target selection. Tumor material and PBMCs of preclinical samples and patients were used for high throughput Next Generation Sequencing. Data were analyzed using an algorithm for mutation detection to identify and select highly immunogenic, tumor-specific, somatic mutations. Immune response against APVAC2 peptides in patients was assessed by intracellular cytokine staining.
Proof-of-concept experiments for the APVAC2 approach have been performed in a melanoma mouse model. Fifty tumor specific DNA mutations have been detected by NGS; 32% of mutations have proven to be immunogenic after peptide vaccination. Patients in the GAPVAC trial displayed a median of 36 (range: 19-84; mean: 42) somatic, non-synonymous mutations within the analysed glioblastoma specimen including known driver mutations (e.g. PIK3CA, IDH1). For every patient the two most promising candidates have been selected, according to the HLA binding prediction, mRNA expression and predicted immunogenicity. Ten patients have been vaccinated with the on-demand manufactured mutation coding peptide vaccines and 11 of the 13 vaccinated mutated 19mers were immunogenic. In addition, the mutational landscape of a tumor sample from relapse after peptide vaccination has been analyzed.
The clinical translation of an immunotherapy with patient individual target identification, selection and on-demand manufactured peptides was successful. As initially shown in mouse models, peptide vaccination is safe and feasible and the mutated antigens induced high immunogenicity in patients with newly diagnosed glioblastoma.

006

HepaVac-101 first-in-man therapeutic cancer vaccine phase I/II clinical trial for hepatocellular carcinoma patients

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HCC is the third leading cause of death from cancer globally with an extremely variable 5-year survival rate. Immunotherapy strategies for HCC may represent a key therapeutic tool to improve clinical

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outcome in HCC patients. The HepaVac-101 phase I/II, first-in-man, single-arm clinical trial is performed as part of the HepaVac project, funded by the European Commission's 7th Framework Program under the Grant Agreement Nr. 602893 (www.hepavac.eu). The HepaVac-101 trial identification numbers are NCT03203005 (Clinical trials.gov) and 2015-003389-10 (EudraCT).
The therapeutic cancer vaccine IMA970A is a multi-peptide-based HCC vaccine composed of 16 newly discovered and overexpressed tumor-associated peptides (TUMAPs) directly identified from resected HCC tissues. Of these TUMAPs, 7 are restricted to HLA-A*02, 5 to HLA-A*24 and 4 to HLA class II. CV8102 is a novel ribonucleic acid (RNA) based immunostimulatory agent inducing a balanced Th1/Th2 immune response. Patients with very early, early and intermediate stage of HCC are enrolled to be treated with a single pre-vaccination infusion of low-dose cyclophosphamide, followed by 9 intradermal vaccinations consisting of IMA970A plus CV8102. The study drugs are applied without concomitant anti-tumor therapy, in order to reduce risk of tumor recurrence/progression in patients having received all indicated standard treatments and without evidence of active disease. The primary endpoints of the HepaVac-101 clinical trial are safety, tolerability, and immunogenicity. Secondary/exploratory endpoints are additional immunological parameters in circulation (e.g. regulatory T-cells, myeloid-derived suppressor cells, impact of the standard therapy on the natural immune response), infiltrating T-lymphocytes in tumor tissue, biomarkers in blood and tissue, disease-free survival/progression-free survival and overall survival. Once safety of this vaccination approach has been established in the first 10-20 patients the addition of a checkpoint inhibitor will be considered. Overall, it is planned to enroll 40 patients. Suitable patients enrolled at Tuebingen are invited to participate in a trial extension investigating an actively personalized vaccine (APVAC). The HepaVac-101 trial is conducted in 6 centers located in 5 European countries. Four centers are actively recruiting patients and one additional site will start enrollment in Feb 2018. As of the time of abstract submission, 4 HCC patients have been screened for HLA haplotype and 1 is eligible for vaccination.

007

Dendritic cell-targeted nano-vaccines synergize with anti-PD-1/anti-OX40 for melanoma treatment

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Immune checkpoint therapy significantly improved the clinical outcome of melanoma treatment. However, results are far from the initially expected. In fact, Programmed cell death protein-1 (PD-1) antibody monotherapy induced effective and durable responses in 30-40% of advanced melanoma patients. Monoclonal anti-OX40, an immune checkpoint stimulator, member of the tumor necrosis factor (TNF) receptor family, has shown modest monotherapy outcomes in clinical trials. Poor clinical results have been associated with complex mechanisms behind antitumor immunity. Currently, it is widely accepted that melanoma therapy will benefit from integrated complementary approaches, which can inhibit tumor immunosuppressive pathways and enhance immunity in an orchestrated manner. We hypothesized that combination therapy with anti-PD-1/anti-OX-40 - to inhibit tumor immunosuppression and to boost T-cell activity, respectively - could be improved by cancer vaccination, by increasing tumor-associated antigen recognition, internalization, processing and presentation to those T cells.
Mannose-poly(lactic-co-glycolic acid)/poly(lactic acid) (man-PLGA/PLA) nanoparticles were produced as dendritic cell (DC)-targeted nano-vaccines by the double emulsion solvent evaporation method. These nano-vaccines were designed to deliver melanoma MART-1 peptide antigens, and the toll-like receptor ligands CpG and MPLA.
Particle internalization by DC was evaluated *in vivo* by flow cytometry. Animals were immunized by subcutaneous injection, while anti-PD-1 and anti-OX40 monoclonal antibodies were administered via

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intraperitoneal injection.
DC-targeted nano-vaccines triggered secretion of inflammatory cytokines and induced cytotoxic T-cell activity against melanoma cells *in vivo*. The combination of DC-targeted nano-vaccines with anti-PD-1/anti-OX40 induced maximal tumor inhibition, leading to 100% of survival 42 days after of tumor inoculation, against 20% obtained for anti-PD-1/anti-OX-40 treatment. In the combination group, 50% of the animals were still alive two months after tumor inoculation, with a high percentage of infiltrating lymphocytes within the tumor.
The synergistic combination of nano-vaccines with anti-PD-1/antiOX40 provide essential insights to devise alternative combination regimens to improve the efficacy of immune checkpoint modulators in melanoma.

008

Molecular mechanism of immune cell activation by therapeutic melanoma vaccine
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Melanoma (MM) is a heterogenous disease that belongs to the most invasive human malignancy with rapidly rising incidence. Recently, significant progress in the development of novel therapeutic modalities of advanced MM was observed. However, not all patients benefit from the therapy. Accordingly, markers for treatment personalization are required to increase the effectiveness. Progress and better understanding of immunotherapeutic approaches brought hope to cure MM We have developed therapeutic gene modified allogenic MM vaccine (AGI-101H) that has been tested since 1997 and resulted in a long-term survival of a substantial fraction of immunized patients.
Our goal is to understand the molecular mechanisms of immune cells that determine response to the AGI-101H treatment. Accordingly, T lymphocyte mRNA expression profiling of long-term surviving patients was carried out. Briefly, PBMCs were isolated from immunized patients (n = 18), from untreated MM patients (n = 13) and healthy controls (n = 8). Untouched T lymphocytes were separated with magnetic beads and total RNA was isolated. The quality and quantity of RNA was verified, and the transcriptome profiling was performed with Affymetrix HG U219 microarray (19285 markers). Differential gene expression (DGE) analysis between groups was conducted and validated with RT-qPCR and FACS. The transcriptomic results were further analyzed with Gene Set Enrichment Analysis (GSEA) tool.
DGE analyses comparing AGI-101H vaccinated (AV) and non-immunized (C) patients revealed 538 differentially expressed genes (DEGs), with 373 downregulated and 165 upregulated in AV (adj p-val < 0.05). Among these 538 markers, the expression of 14 (with |log2FC| > 1) is now being validated with RT-qPCR and FACS. GSEA analysis revealed significant enrichment of *"TNFa_signaling_via_NFkB"*, *"TGFb_signaling"* and *"G2/M_checkpoint"* hallmark processes (MSigDB Hallmark gene sets), that confirms significant activation of anti-tumor response and indicates activation of T cell differentiation into functionally distinct lineages.
Transcriptome profiling of T lymphocytes in long-term surviving MM patients immunized with AGI-101H vaccine may help in understanding the molecular mechanism of activation of immune cells by AGI-101H vaccine and characterize the features of immune cells that determine the clinical response to the treatment.

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A cancer vaccine targeting many neo-antigens is required for efficient eradication of large tumors

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T cells targeting cancer neoantigens play a crucial role in tumor control, as demonstrated by current immunotherapies based on checkpoint inhibitors (CPI). Here we developed a novel neoantigen cancer vaccine based on a genetic viral vector, namely Great Ape Adenovirus (GAd), able to deliver a large number of cancer-specific single nucleotide variants (SNVs). In murine tumor models, we demonstrated induction of potent CD8 and CD4 interferon-γ (IFN-γ) producing T cells upon vaccination. Combined treatment of vaccine and anti-PD1 induces tumor growth inhibition in vivo, with half of treated mice achieving eradication of large established tumors. Synergic activity between anti-PD-1 and vaccine was not observed with a vaccine encoding a limited number of neoantigens. Effectiveness of the vaccination in animals with high tumor burden requires concomitant administration of a checkpoint inhibitor and correlates with breadth of vaccine induced T cell responses.

010

Successful completion of the Mutanome Engineered RNA Immuno-Therapy (MERIT) project

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The treatment of triple negative breast cancer (TNBC) is hampered by the lack of established therapeutic targets such as hormone receptors or HER-2. Chemotherapy and radiotherapy is the standard of care, yet survival rates in TNBC remain poor. The **Mutanome Engineered RNA Immuno-Therapy (MERIT)** project was implemented by a consortium of five European partners in academia and industry and is validating a highly innovative, individualized mRNA based immunotherapy approach for TNBC patients.
Besides a broad preclinical program the consortium has set up a clinical workflow and trial design, which covers the drug development cycle from target identification and selection to GMP manufacturing and drug release for each individual patient. Two novel approaches comprising highly personalized as well as on-demand manufactured RNAs for vaccine-based immunotherapy for TNBC patients are investigated in a phase I trial. Patients in ARM1 receive eight vaccination cycles with a personalized combination of shared tumor-associated antigens (TAAs), which were selected based on each patient tumor's antigen-expression profile out of a WAREHOUSE of pre-manufactured mRNA vaccines. Patients in the second study arm receive the personalized mRNA WAREHOUSE vaccine followed by eight vaccination cycles of an on-demand manufactured mRNA MUTANOME vaccine encoding unique neo-epitopes of the individual patient identified by NGS. The mRNAs are administered intravenously as a lipoplex formulation.
A phase I trial in four European countries assesses the feasibility, safety and biological efficacy of this

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personalized immunotherapy. Until the end of the MERIT project period, ten patients were vaccinated with RNA WAREHOUSE vaccines coding for shared TAAs. Preliminary data show that the RNA-WAREHOUSE approach can be applied safely, is well-tolerated and induces detectable immune responses in TNBC patients. The clinical trial is ongoing and the first MUTANOME RNAs for ARM2 patients have been produced.
In addition a broad preclinical program has been completed. (i) A computational medicine platform was implemented that allows for rapid identification of immunogenic shared and mutated antigens in TNBC patients, (ii) an associated biomarker study was set up to identify molecular and immunological signatures that correlate with clinical events following treatment and (iii) synergistic compounds as well as different vaccination protocols were investigated to enhance the efficacy of the mRNA vaccine. With the successful completion of the MERIT project, we gained information about the safety and feasibility of a highly innovative RNA-immunotherapy approach and by using an innovative trial concept we seek to increase the clinical benefit for TNBC patients.

The MERIT project was funded by the European Union Framework 7 Program.

011

Bifunctional RNA-nanoparticles to enhance dendritic cell activation and enable MRI-based detection of dendritic cell migration to lymph nodes

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Background: Cancer vaccines are a promising approach to personalized cancer immunotherapy, but the lack of meaningful biomarkers of patient response to treatment limit their development. We recently reported in a randomized, double blind, placebo controlled trial that RNA-pulsed dendritic cells (DCs) prolong progression free and overall survival in patients with glioblastoma (Mitchell et al, *Nature* 2015). Furthermore, we demonstrated that DC migration to lymph nodes assessed by SPECT/CT imaging strongly correlates with clinical outcomes. While this finding may provide a novel imaging biomarker for response to DC vaccines, the complexity and regulatory requirements of nuclear medicine-based imaging of radiolabeled cells limits widespread utilization of this technique. We have therefore developed bi-functional RNA-loaded nanoparticles (RNA-NPs) to further enhance DC migration to lymph nodes and track migration *in vivo* using a widely available MRI-based imaging modality.
Methods: Cationic liposomes with iron oxide nanoparticle cores were incubated with mRNA. The resulting iron oxide-loaded RNA-NPs (IO-RNA-NPs) were used to transfect DsRed+ DCs *ex vivo* in the presence of a magnetic field. IO-RNA-NP-loaded DCs were then injected intradermally into C57Bl6 mice and tracked noninvasively with T2-weighted 11T MRI. MRI intensity was correlated with Prussian blue staining for iron oxide content and flow cytometry for absolute counts of DsRed+ cells in each lymph node.
Results: The presence of iron oxide in RNA-NPs did not significantly modify particle characteristics including size, charge, RNA-binding capacity, and transfection of DCs. Additionally, inclusion of iron oxide within RNA-NPs enabled magnetically enhanced RNA delivery and transfection efficiency through application of external magnetic fields. Compared to RNA electroporation, IO-RNA-NP loading enhanced production of antiviral cytokines (IFN-alpha) and DC migration to lymph nodes. IO-RNA-NPs also produced a reduction in T2-weighted MRI intensity and an increase in MRI-detected lymph node size that correlated directly with the number of iron oxide loaded cells in treated lymph nodes.
Conclusions: This data suggests that IO-RNA-NPs enhance DC activation and allow noninvasive cell tracking with MRI. Future work will consider effects of IO-RNA-NPs on antitumor immune responses and the utility of MRI-detected DC migration as a biomarker for vaccine efficacy.

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E6/E7 RNA$_{(LIP)}$: An RNA cancer vaccine for treatment of patients with HPV16-positive malignancies

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Human papillomavirus (HPV) has emerged as a major risk factor for Head Neck squamous cell carcinoma (HNSCC) and the incidence of HPV-positive HNSCC continues to rise. Standard treatment of HNSCC given with curative intent causes substantial and long-term physical and functional impairments, but nonetheless, approx. 50% of patients die of their disease. Alternative treatments are urgently needed to improve survival but also to reduce treatment-associated morbidity. Both $CD8^+$ and $CD4^+$ T cells are important for viral clearance and regression of HPV-positive premalignant lesions; density of tumor-infiltrating lymphocytes (TILs) is a strong predictor of the outcome of HPV-positive oropharyngeal cancers. Consequently, therapeutic cancer vaccines hold great promise to achieve long-term disease control in HPV-positive malignancies if they generate tumor antigen-specific and durable anti-cancer immunity that can recognize viral antigen at the tumor site.
Recent advances in the systemic delivery of antigen-encoding lipid-complexed RNA (RNA$_{(LIP)}$) allow efficient targeting and RNA delivery to secondary lymphoid organs body-wide with early data supporting substantial immunological and clinical effects. With E6/E7 RNA$_{(LIP)}$, BioNTech has developed a promising immunotherapeutic approach to address a high unmet medical need. E6/E7 RNA$_{(LIP)}$ is the first systemically administrable therapeutic cancer vaccine based on two lipid-complexed RNA drug products targeting the well-characterized HPV16-derived viral antigens E6 and E7. Preclinical studies in two independent syngeneic mouse tumor models demonstrated that vaccine treatment significantly increased the frequency of TILs, leading to strong tumor microenvironment polarization and consistent regression of large established tumors.
E6/E7 RNA$_{(LIP)}$ is currently evaluated in a multi-center, first-in-human phase I/II trial in patients with HPV16-positive HNSCC and other HPV16-positive cancers (Sponsor: University Hospital of Southampton NHS Foundation Trust; EudraCT No.: 2014-002061-30). Objectives of the trial are to study safety, tolerability, and immunogenicity of the novel immunotherapeutic approach. Where measurable tumor is present clinical response rate and expansion of HPV-specific TILs will be evaluated. Here we present the preclinical proof-of-concept data as well as preliminary data from the ongoing clinical trial.

013

A novel rMVA combination immunotherapy triggers potent innate and adaptive immune responses against established tumors

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Virus-based vaccines and appropriate costimulation enhance potent antigen-specific T cell immunity

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against cancer. However, the tumor microenvironment exerts intrinsic and extrinsic mechanisms to evade tumor destruction. In the present study we exploit both innate and adaptive immune responses triggered by a novel recombinant modified vaccinia virus Ankara (rMVA) encoding costimulatory CD40L against solid tumors in combination regimes to overcome tumor-induced resistance to immunotherapy.
Therapeutic treatment with rMVA-CD40L resulted in strong antitumor effects in unrelated established tumor models. Tumor infiltration was composed of non-exhausted, antigen-specific $CD8^+$ T cells with proliferative capacity after rMVA-CD40L immunization.
Strikingly, this antitumor effect was entirely dependent on $CD8^+$ T cells, however only partially reduced in $CD8\alpha^+$ DC-deficient mice. Furthermore, rMVA-CD40L-induced tumor control did not depend on the cytosolic DNA sensor STING.
Interestingly, rMVA-CD40L induced strong NK cell activation and thereby potent antibody-dependent cell cytotoxicity (ADCC) against tumor-associated antigen (TAA) targeting antibodies. Hence, the combination of TAA targeting antibodies and rMVA-CD40L resulted in increased therapeutic antitumor efficacy.
We describe a novel and translationally relevant therapeutic synergy between viral vaccination and CD40L costimulation. Taking advantage of intrinsic MVA-induced NK cell activation and improved NK cell function by CD40 ligation, we show strengthened antitumor immune responses when both rMVA-CD40L-induced innate and adaptive immune mechanisms are exploited by combining immunotherapeutic regimes. This finding has a direct potential impact on clinical trials where TAA targeting antibodies are currently under evaluation.

014

A first-in-human phase I/II clinical trial assessing novel mRNA-lipoplex nanoparticles encoding shared tumor antigens for immunotherapy of malignant melanoma

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Therapeutic vaccination with tumor antigen-encoding RNAs is being investigated in various clinical trials. Typically, the RNA vaccine is administered intradermally, subcutaneously or intranodally with the intention to get expression of the encoded antigens in local antigen-presenting cells (APCs).
We have developed a novel class of RNA-lipoplex ($RNA_{(LIP)}$) immunotherapeutics for intravenous application, which allow systemic targeting of APCs. $RNA_{(LIP)}$ is a novel nanoparticulate formulation of lipid-complexed mRNA which selectively delivers the functional mRNA to APCs in lymphoid compartments body-wide for efficient mRNA uptake and expression of the encoded antigen by APCs. Moreover, this formulation has intrinsically strong adjuvant activity, mimics a systemic viral infection and induces synchronized activation of potent adaptive as well as type-I-IFN-mediated innate immune responses (Kranz et al., Nature 2016).
The first-in-human phase I/II dose escalation Lipo-MERIT trial (NCT02410733) conducted in four German study centers assesses the safety, tolerability, and biological efficacy of $RNA_{(LIP)}$

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immunotherapy in patients with stage IIIB/C and IV melanoma. This trial is the first to investigate intravenous administration of a RNA-based cancer vaccine. Following antigen expression stratification on routinely collected tumor samples, eligible patients are treated with repeated dosing of the tetravalent Lipo-MERIT vaccine composed of $RNA_{(LIP)}$ products encoding the shared melanoma-associated antigens NY-ESO-1, tyrosinase, MAGE-A3, and TPTE. Pharmacodynamic activity and immunogenicity of the vaccine is investigated by concerted immune monitoring and correlative biomarker studies. Clinical activity is assessed following imaging according to irRECIST1.1.
As of March 2018, >60 patients have been treated with escalating or constant dosing under the guidance of an independent data safety and monitoring board. The Lipo-MERIT vaccine was generally well-tolerated and no dose-limiting toxicities (DLTs) were observed so far. Further patient enrollment is continuing.
Preliminary results from immunological assessments of the first patients treated are indicative of a high rate of vaccine-induced immunity and will be presented.

015

Novel linear doggybone™ DNA vaccine induces anti-tumour immunity via STING and independent of TLR9

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DNA vaccine encoding target antigens is an attractive modality to induce immunity against cancer, which has already shown promising results in clinical setting. Conventional DNA vaccine utilises a plasmid that encodes the antigen of interest and includes bacterial immunostimulatory CpG motifs recognised by Toll-like receptor 9 (TLR9). Recently a novel DNA vaccine using doggybone DNA (dbDNA™), has been developed without the use of bacteria. The cell-free manufacturing process relies on Phi29 DNA polymerase to amplify a plasmid template, and uses protelomerase TelN to complete individual closed linear DNA. The final DNA product is simply composed of the sequence encoding the antigen of interest, a promoter and a poly A tail, but lacks bacterial sequences, including antibiotic resistance genes.
The ability of dbDNA™ vaccine to induce an adaptive immune response with and without *in vivo* electroporation was compared with plasmid DNA vaccine, using clinically relevant oncotargets E6 and E7 from HPV16. Despite the inability to trigger TLR9, dbDNA™ induced similar levels of antigen specific $CD8^+$ T cells, Th1 $CD4^+$ T cells and antibody responses as plasmid DNA vaccine. dbDNA™ also showed similar ability to suppress the established TC-1 tumors. Furthermore we demonstrated that dbDNA™ vaccine was recognised via cytosolic sensing pathways involving STING, with the induction of type I interferons and Th1-polarizing cytokines.
Taken together, dbDNA™ is a highly attractive novel DNA vaccine platform to induce anti-cancer immunity.

016

Nucleic Acid based TRL ligand loaded nanocomplexes act as standalone antitumor agents

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Toll-like receptor 3 (TLR3), TLR7/8 and TLR9 senses nucleic acid-based agonists. These ligands have great potential as tumor immunotherapy agents.

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When triggered by these ligands antigen presenting cell (APC) functions are boosted causing improved cytotoxic T-cells (CTL) activity. Notably, subversion of immunosuppressive microenvironment mediated by tumor micro niche is reversed by the action of administered immunostimulatory ligands. Yet when these agents were given *in vivo*, they are rapidly digested by nucleases, and could be adsorbed by circulating serum proteins hampering *in vivo* therapeutic index. We designed a CpG ODN sequence that readily conforms self-assembled nanoparticle formation. (NP-ODN hereafter). In addition, β-glucan based polysaccharide nanocomplexes with single, dual or triple nucleic acid TLR ligand combinations were prepared (PS/TLRL hereafter). Following in vitro activity characterization these nanoparticles were tested as potent standalone anti-cancer immunotherapeutic agents to control established hepatocellular or thymoma tumor growth.
HUH7, hepatocellular carcinoma and EG7 thymoma xenografts were xenografted in athymic nude and C57BL/6 mice (5-10/group, $5x10^6$ cancer cells/animal), respectively. Three doses of 100µg NP-ODN or PS/TLRL nanocomplexes on alternating days were peritumorally administered following palpable tumor formation. Tumor dimensions were recorded daily and calculated as in mm^3.
Untreated or free ODN treated nude mice at the end of d=30 developed a tumor volume ~2000±150 mm^3 and 1300±235mm^3 respectively. A >95% of tumor size reduction was observed in mice treated with NP-ODN (180±50 mm^3). Of note, a 40% complete regression was observed in NP-ODN treated group whereas there was no complete remission in those animals that received free ODN.
The antitumor effect of natural PS/CpG-ODN nanocomplexes were more effective than untreated or only PS treated or only non-complexed ODN treated mice that was inoculated with EG7 cells ($5x10^6$/animal). Almost >50% tumor size reduction was observed in mice that had nanocomplex. Starting from day 16 and onwards this profile remained same until the cessation of experiment on d=30.
Treating mice either with self-nanoparticle forming CpG ODN or PS/CpGODN nanocomplexes demonstrated a significant tumor regression in two different established tumor models.
The mechanism responsible from this effect could be attributed to a more pronounced natural killer cell activation in hepatocellular carcinoma model, and effective APC priming leading to augmented CTL activity in thymoma model. Dissection of various cell types contributing CpG-nanoparticle mediated tumor regression is underway.

017

Discovery and vaccine development for hepatocellular carcinoma - HEPAVAC

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Hepatocellular carcinoma (HCC) is among the most frequent and lethal cancers in the world and a major health issue on a global scale. Unless surgically resected in early stages, treatment attempts with curative intent are rarely successful and adjuvant treatment options for high risk patients are lacking. Since effective additional treatment options for HCC are sought-after. The HEPAVAC

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Consortium consisting of 9 European Partners from academia and the biotech industry therefore aims to develop a highly innovative, novel cancer vaccine approach, consisting of tumor-specific peptides and a novel RNA-based adjuvant. The consortium is supported by the European Commission's 7th framework program (www.hepavac.eu).
From 126 primary HCC samples collected for discovery, 32 HLA-A*02 and/or A*24 positive samples were selected for analysis. From these samples, HLA-bound peptides were eluted after immunoaffinity chromatography, identifying 7262 and 3297 unique tumor-associated peptides (TUMAPs) for HLA-A*02/ A*24 respectively by mass spectrometry. Using the XPRESIDENT® discovery platform, 34 HLA-A*02 and 36 HLA-A*24 restricted peptides were selected and assessed for immunogenicity. Candidate peptides were tested for *in vitro* immunogenicity, using standardized *in vitro* assays based on priming and expansion of isolated human CD8+ cells from healthy HLA-matched donors, confirming 26 HLA-A*02 and 27 HLA-A*24 restricted peptides as immunogenic.
The target discovery approach was complemented by the identification of HLA class II binding peptides on HCC samples as well as on CIITA-transfected cell lines by mass spectrometry. Through comprehensive analyses, 4 HLA-DR TUMAPs were identified by comparative expression in fresh HCC tumor samples and CIITA-transfected HCC tumor cell lines and chosen to complement the multi-peptide vaccine, aiming for a balanced Th1/Th2 immune response.
The final *off-the-shelf* multi-peptide vaccine, combining five HLA-A*24-, seven HLA*A02- as well as 4 HLA-DR restricted peptides, was manufactured in the form of synthetic peptides according to GMP guidelines, formulated and released. The HepaVac-101 phase I/II, first-in-man clinical trial for HCC patients in an adjuvant setting is currently recruiting patients in 5 European countries. Study treatment encompasses the therapeutic multi-peptide cancer vaccine (IMA970) as well as the novel RNA based immunostimulatory agent CV8102, together with single low dose conditioning with cyclophosphamide.

018

Retrospective analysis of neoantigen prediction methods
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Neoantigens arise from somatic tumor mutations, which are translated into tumor specific proteins, and can be recognized by the patient's adaptive immune system. With the success of checkpoint inhibition based therapies neoantigens became a focus of attention, as the existence of such has been shown to be predictive for therapy success. Moreover, neoantigens are useful as a biomarker, e.g. being correlated with long-term survival in pancreatic ductal adenocarcinoma. Technological breakthroughs of the last decade, namely next generation sequencing, allow the genome wide study of neoantigen profiles in patient cohorts, enabling clinical studies for the use of neoantigens as vaccine targets. Several such studies are currently conducted, requiring a truly multidisciplinary production process, including genomics, bioinformatics and GMP manufacturing. The challenges for the bioinformatics process include a reliable mutation detection and the selection of potentially valuable targets for a vaccine, which is usually based on peptides or oligonucleotides. Several approaches for the target selection have been proposed. Here we present a retrospective analysis of published data, including results of the IVAC Mutanome trial (ClinicalTrials.gov Identifier: NCT02035956), using several published neoantigen prediction algorithms.

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Modulating T cell immunity in tumors by targeting PD-L1 and neoantigens using a live attenuated oral *Salmonella* platform

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Significant progresses have been achieved recently in cancer vaccines that aim at engaging or reengaging tumor directed T cells, in particular in the rapidly growing field of personalized immunotherapy. Yet novel immunization solutions to produce effective tumor-associated (neo)antigens T cell responses while simultaneously overcoming the immunosuppressive tumor microenvironment are urgently needed. We are developing a unique and versatile oral T-cell vaccination platform based on the FDA-approved live-attenuated *Salmonella* Typhi strain Ty21a vaccine Vivotif®, capable of delivering tumor-associated antigens encoded in DNA expression construct to the gut-associated lymphoid tissue, breaking immune tolerance and inducing anti-tumor immunity.
This study summarizes the immunogenicity and antileukemia efficacy of VXM10 vaccines based on the live-attenuated *Salmonella* Typhimurium strain SL7207, transformed with a eukaryotic expression plasmid encoding the murine programmed death-ligand 1 (PD-L1) protein. It also describes the systemic immunogenicity of *Salmonella* based polyepitope oral vaccines, supporting the design of *Salmonella* based neoantigen vaccines.
The antileukemia activity of VXM10 was evaluated in the FBL-3 model of leukemia, in which the tumor cells express high levels of PD-L1. Oral administration of VXM10 produced a strong anti-tumor effect in the FBL-3 leukemia model, with 100% of surviving animals 80 days after leukemia challenge in the highest dose groups. In contrast, administration of the empty vector control did not show any anti-cancer effect. Moreover, 100% of long-term surviving mice resisted re-challenge with FBL-3 cells, demonstrating that vaccination with VXM10 generated a potent memory T cell response against the leukemia. Importantly, full leukemia control was achieved in both prophylactic and therapeutic settings. The anti-tumor effect observed in these experiments was as strong as in previous experiments using a *Salmonella* Typhimurium transformed with a plasmid encoding Wilm´s tumor 1 (WT1). The anti-tumor efficacy was accompanied by an increased systemic antibody response, and the activation of T cells directed against PD-L1 epitopes.
Finally, different polyepitope vaccines encoding model epitopes from VEGFR2, Mesothelin, WT1, CEA, and Ovalbumin, induced a significant systemic immunogenicity for up to 6 out of 9 epitopes, 10 days after vaccination of healthy C57BL/6 mice via the oral route, as measured in the spleen by flow cytometry using peptide-MHC class I pentamers.
These studies demonstrate that the *Salmonella* platform can be used to generate anti PD-L1 antibody and T cell responses, as well as CD8-positive T cell responses against a majority of MHC class I epitopes of a polyepitope construct. We are now designing a clinical neoantigen-based vaccine that employs the concepts presented.

020

Enrichment of gene modified cellular melanoma vaccines with melanoma stem cells or inducible pluripotent stem cells increases immunogenicity and clinical effectiveness in mice

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The aim of the study was to verify the hypothesis that tumor vaccines containing Cancer Stem Cells

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(CSC) exhibit increased immunological and clinical efficacy than vaccines based on differentiated cells only. For the CSC-based vaccines construction melanospheres formed by B16F10 cells cultured in suspension in two different media (referred to as MI or MII) were used. They showed melanoma stem cells-like phenotype (low MITF and tyrosinase, high VEGF, CD44 expression and high ALDH1 activity). The third vaccine consisted of murine inducible pluripotent stem cells (miPSC) admixed with B16. $1x10^6$ vaccine cells (MI, MII or miPS) were admixed with $0.5x10^6$ H6 (hyper-IL-6, fusion protein composed of IL-6 and IL-6 soluble receptor) expressing B16 cells (B16H6). Control groups were immunized with $1x10^6$ B16 + $0.5x10^6$ B16H6 or $1x10^6$ B16 or PBS. The phenotype of immune cells infiltrating the vaccine or tumor in matrigel, and proinflammatory cytokines produced in these sites were analyzed. Mice immunized with MI/H6, MII/H6, and miPSC/H6 containing vaccines demonstrated higher activation of the local immune response in vaccination site and tumor microenvironment compared to B16H6 and controls. Significantly higher number of infiltrating dendritic cells (DC), monocytes, (natural killers) NK cells, and lower number of myeloid derived suppressor cells (MDSC) and $Foxp3^+$ T regulatory cells were observed. Higher level of pro-inflammatory cytokines: INFγ and IL-12 was observed. In-vitro re-stimulation of splenocytes from immunized mice with lysates from the tumor and vaccines cells demonstrated increased proliferation of CD4+ cells in all study groups. Increased production of IL-2 was seen in all vaccinated mice, especially in the miPSC/H6 group. Moreover, in the miPSC/H6, MI/H6 and MII/H6 groups, higher IL-10 level was seen. In sera from MI/H6 and MII/H6 immunized animals, B16-specific IgG antibodies were found. The therapeutic potential of the vaccines was assessed in localized prophylactic/therapeutic melanoma mouse model. MI/H6, MII/H6 and miPSC/H6 vaccines inhibited development of melanoma tumors. The strongest effect was observed in miPSC/H6 immunized mice - (70% of mice lived more than 120 days without progression). In the MI/H6 and MII/H6 vaccinated groups, 50% of the mice lived for more than 120 days without disease progression. The median OS in MI/H6 and MII/H6 was 85.5 and 87 days. In control groups (PBS, B16) 15 days after tumor cell injection, all mice progressed. Immunization with irradiated miPSC alone had no effect on tumor growth.
In conclusion, CSC- and miPSC-based cancer vaccines have been shown to be more effective in inducing a specific immune response and inhibiting local immunosuppression compared to the mouse melanoma vaccine. These vaccines also showed greater anti-melanoma therapeutic potential. It is possible that added cells provided extra adjuvant signals and CSC antigens.

021

The immunopeptidomic landscape of breast cancer

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Significant recent advances in cancer immunotherapies have led to long-term remission in patients with melanoma and non-small cell lung carcinoma. These therapies are currently tested in other cancer entities and it becomes evident that cancer immunotherapy is not beneficial for every patient. By knowing the antigenic repertoire presented by HLA molecules on breast cancer cells, patient-individualized immunotherapies could be tailored to target antigens exclusively-presented on cancer cells.
Therapeutic decisions in breast cancer are based on molecular classification into four major tumor subtypes: luminal A, luminal B, Her2-positive, and triple negative. Treatment options include surgical resection, radiation, chemo- and endocrine therapy. The Her2 subtype treatment is supplemented with therapeutic antibodies targeting Her2, which are the only approved immunotherapies in breast cancer so far.
Comparative ligandome profiling between tumor and benign samples allows for confident identification of tumor-associated antigens (TAA). In this study we employed mass spectrometric characterization of

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the immunopeptidome of 12 primary breast cancer tissues. Thus, we were able to identify more than 15,000 HLA class I and 20,000 HLA class II peptides. Benign HLA ligandome data was obtained by mapping 230 benign tissue samples including mamma, kidney, liver, and brain from 15 donors. These benign tissues were obtained from autopsy specimens and have been shown to represent an adequate database for HLA-matched comparisons, as they comprise over 88,000 unique HLA class I peptides and 157,000 HLA class II peptides.
Consequently, we were able to define a set of TAAs in breast cancer patients, which can be targeted by various immunotherapeutic strategies, such as peptide vaccination. To further refine the set of naturally presented TAAs, we will expand the number of breast cancer specimens analyzed. Thus identified TAAs will be prioritized by comparison with natural HLA ligands identified from circulating breast cancer stem cells.

022

Multivalent polymeric nanoparticles for combinatorial innovative colorectal cancer immunotherapy and immunomodulation

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Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth cause of cancer death worldwide, being respossible for approximately 700,000 deaths per year. Almost 1/5 of CRC patients are diagnosed with the metastatic disease and present a very low 5-years survival rate. Immunotherapeutic approaches have shown very positive outcomes in other disseminated malignant diseases. However, an effective strategy against the heterogeneous population of cancer cells requires a combinatorial strategy to modulate different cells and mechanisms involved in tumor growth, proliferation and metastization. This work focused on the development of a combinatorial multivalent nanoplatform for CRC immunotherapy and immunomodulation based on the design of polymeric nanoparticles (NP) able to deliver a combination of CRC-associated antigen, adjuvants and gene regulators according to targeted cells, dendritic cells (DC) and CRC cells.
Poly(lactic-co-glycolic) (PLGA)-based NP were prepared by the double emulsion (w/o/w) solvent evaporation method. NP physicochemical characterization was performed in terms of size, zeta potential and surface morphology. CRC antigen loadings were quantified by fluorescence. Immature DC (ATCC® CRL-11904™) were used to evaluate the *in vitro* NP cytotoxicity by the propidium iodide assay, as well as NP cellular uptake profile by flow cytometry. *In vivo* biodistribution assay of plain NP was also performed using the IVIS Lumina® Bioimaging system. NP uptake *in vivo* by myeloid antigen presenting cells and the expression of maturation and co-stimulatory molecules at the surface of these cells sorted within draining lymph nodes, were also evaluated by flow cytometry.
PLGA-based NP presented a mean size diameter close to 200 nm, with low polydispersity index (≤0.200), a surface charge close to neutrality, as well as, a spherical shape and smooth surface. These multivalent delivery systems presented high loadings for antigen and adjuvants. No cytotoxic effect was observed on immature DC up to 48 h of incubation. NP were extensively internalized by immature DC *in vitro* after 48 h incubation, and by migratory DC *in vivo* 17 h after animal immunization. *In vivo* real-time monitoring of NP accumulation in mice whole bodies and dissected organs (liver, spleen, kidneys, heart, lungs and lymph nodes) showed a fluorescent signal at 17 h close to the site of immunization and in the lymph nodes. No significant differences in the expression of the co-stimulatory CD80, CD86 and MHC class I markers on $CD11b^{+}CD11c^{+}MHCII^{+}$ population at lymph nodes were observed among different polymeric combinations upon mice immunization with NP carrying CRC antigen and adjuvant.

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According to NP physicochemical characteristics, internalization and biodistribution patterns, this innovative nanoplatform can lead to a safe multivalent nanomedicine able to modulate dendritic cell activity and T cell expansion against tumor cells expressing entrapped antigens.

023

Ibrutinib in combination with T-cell based immunotherapy in chronic lymphocytic leukemia (CLL) - mass spectrometry-based HLA ligandome analysis of primary CLL cells approves stable presentation of leukemia-associated antigens

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The small molecule ibrutinib targets chronic lymphocytic leukemia (CLL) by inhibition of the Bruton tyrosine kinase (BTK), with response rates up to 63%. Furthermore, several studies have demonstrated a positive effect of ibrutinib on T-cell based immune responses like enhancing the T-cell repertoire of CLL patients as well as improving antigen presentation. For the rational combination of T-cell based immunotherapy with ibrutinib, it is thus of great importance to thoroughly characterize the effects of the BTK inhibitor not only on effector cells, but also on the antigenic landscape of the target cells.
Here we present a mass spectrometry-based study which longitudinally maps the HLA-presented immunopeptidome and in particular CLL-associated antigens of primary CLL cells under *in vitro* ibrutinib treatment. Furthermore, the impact of ibrutinib on peptide-specific T-cell responses was analyzed.
We quantified HLA surface expression on primary $CD19^{+}CD5^{+}$ CLL cells at t_0, t_{24h} and t_{48h} after incubation with 1 µM ibrutinib. With regard to HLA class I and II expression, no marked impact of ibrutinib was observed (mean fold-change of 0.97 for class I, mean fold-change of 0.77 for class II, t_{48h}) with absolute molecule counts ranging from 100,000-200,000 class I and 60,000-170,000 class II molecules/cell.
Implementing label-free quantification, we assessed the HLA class I ligand presentation during *in vitro* ibrutinib incubation. We observed a higher plasticity of the HLA ligandome over time compared to treatment with ibrutinib. 4.99% of HLA ligands showed significant modulation (fold change ≥4, p≤0.01) after 24h of cultivation, whereas only 0.16% of the ligands were modulated upon ibrutinib treatment. At t_{48h} similar proportions of modulation were observed with 9.45% of HLA ligands significantly altered in their abundance over time, while ibrutinib treatment only resulted in 1.85% modulated ligands.
Out of the 1,994 different HLA class I ligands representing 1,723 source proteins identified on CLL cells by mass spectrometry, we were able to detect 18 (35.3%) of the HLA-matched CLL-associated epitopes described in previous studies (Kowalewski *et al.* PNAS 2015). Importantly, these CLL-associated antigens showed robust presentation under *in vitro* ibrutinib treatment.
Furthermore, we examined the effect of ibrutinib on effector cells by analyzing T-cell responses in PBMC samples of CLL patients against a viral peptide mix using IFNγ-ELISpot assays and intracellular cytokine stainings. Importantly, no impairment of absolute CD4 and CD8 T-cell count, peptide-specific T-cell response as well as T-cell functionality could be detected.
Taken together our study provides direct insights into the impact of ibrutinib on the HLA ligandome of primary CLL cells as well as on effector cell function. We were able to show that *in vitro* ibrutinib has no relevant influence on the HLA-presented peptidome of CLL cells as well as on peptide-specific T-cell response.

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Nanoparticle-based vaccine improved OX40 anti-tumor efficacy in breast cancer

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Cancer vaccines are promising alternatives for cancer treatment. However, limited effect on tumor regression has been obtained due to multiple tumor immune evasion mechanisms. OX40 is an immune checkpoint co-stimulator expressed by activated cytotoxic T cells and regulatory T cells. It triggers the expansion and trafficking of effector T cells, while overcoming the suppression of CD4+ T cells by inhibiting FoxP3, TGF-beta and IL-10 expression. However, limited outcomes have been obtained with anti-OX40 agonists at clinical settings. This study characterized the immune-mediated antitumor responses induced by a multifunctional nano-vaccine combined with anti-OX40 in a breast cancer mouse model.
Poly(lactide acid) (PLA) nanoparticles (NP) were synthesized by the double emulsion solvent evaporation method to incorporate α-lactalbumin, as breast cancer antigen, and the toll-like receptor ligands (TLRl) CpG and Poly (I:C). To potentiate the delivery of these TLRl to tumor microenvironment, NP surface was modified by hyaluronic acid to target CD44 overexpressed on 4T1 breast cancer cells. NP size, surface charge (ZP) and morphology were analyzed by Dynamic Light Scattering, Laser Doppler Electrophoresis and Atomic Force Microscopy, respectively. Antigen and adjuvants entrapment efficiencies (EE) were quantified by HPLC and Oligreen® reagent, respectively. Cell viability was assessed by Alamar Blue® assay. NP internalization and DC activation profile were evaluated i*n vivo* by flow cytometry. The immunotherapeutic potential of our multifunctional nano-vaccines was assessed, isolated and in combination with anti-OX40, in 4T1 murine model.
NP presented a mean diameter close to 200 nm with low polydispersity index (PdI) values (≤0.16), surface charge close to neutrality, and EE > 85%. NP showed no negative effects on the viability of DC after 72 h of incubation, even at high NP concentrations (up to 1 mg/mL). Targeted PLA NP, labeled with rhodamine, were extensively taken up by DC, but also by 4T1 breast cancer cells. Nano-vaccines induced the DC activation and maturation, significantly increasing the expression of CD40, CD80, and CD86 surface markers. A noteworthy tumor remission was observed in tumor-bearing animals treated with the multifunctional nano-vaccine combined with anti-OX40. These animals presented a long-term survival, with a tumor volume 4-fold lower than that obtained in OX40-treated mice.
This study reveals the synergy between a multi-targeting nano-vaccine and anti-OX40 in breast cancer, providing important insights for the establishment of novel combination regimens against this poorly immunogenic cancer.
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025

Analysis of the human tumor HLA peptidome based on patient derived xenografts in mice

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The direct biochemical HLA peptidome analysis provides very reliable information about the presentation levels and patterns of neoepitope HLA peptides, by the tumor cells. Using this information, one can select the best-presented neoepitope peptides for designing and prioritizing a vaccine suitable for the patients.
Here we describe the use of patient derived xenografts (PDX) tumor tissue models as a source for HLA peptidome analysis. PDX tumors are already used for testing different anti-cancer drugs, and as a source for large amounts of tumor tissues that maintain closer similarities to the original tumors and their microenvironments, relative to the in-vitro cultured cells. In this research, our goal was to establish PDX mice as an effective model for cancer specific HLA peptides antigen detection in order to implement them in the future into the clinical pipeline of personalized cancer immunotherapy.
We used immunoaffinity purification to recover the HLA molecules from the PDX tumors, followed by capillary chromatography and tandem mass spectrometry (LC-MS/MS) analysis of extracted HLA peptides. We compared the original patients' tumor tissues to the same tumors grown in PDX mice. The HLA peptides were identified using databanks of all human protein sequences, supplemented with sequences of the mutated proteins, as elucidated from exome data of the tumors.
We demonstrate here that the PDX tissues are a reliable source for discovery of large HLA peptidomes, larger than obtainable from the limiting amounts of original tumors. The HLA peptidomes of multiple PDX tumors grown in separate mice and originating from the same human tumors, were very similar, even after three generations of re-grafting. Furthermore, the HLA peptidomes of the PDX were very similar to those of the original tumors. Between 50% to 95% of the original tumors' HLA peptides were also detected in their derived PDX tumors. The majority of the peptides fitted the patient´s HLA sequence motifs and peptides length distributions, supporting the conclusion that these are likely true human HLA peptidomes. Importantly, three new neoantigens HLA peptides were detected in the PDX tumors, which were not detected in the original tumors. These neoantigens fit the patients HLA sequence motif, and therefore can be used as a proof of concept for the implementation of the PDX mice models into the clinical pipeline of personalized cancer immunotherapy.

026

Pseudocowpox (PCPV): a viral vector for cancer immunotherapy
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Engineered viral vectors are effective approaches to stimulate anti-tumor immunity, and change the tumor immune environment. Several viruses and strains have been developed to express tumor antigens and cytokines, and corresponding products are in advanced clinical trials. However, novel viral strains with improved immunogenic properties are sought. In this perspective, we screened a variety of poxviridae potentially usable in humans: Cowpox (CPX), Pseudocowpox (PCPV), Parapoxvirus Ovis (ORF), Myxoma virus (MYX), Swinepox (SWP), Yaba-like disease virus (YLDV), Cotia virus (CTV), and compared them to the well-established vaccine strain Modified Virus Ankara (MVA), and oncolytic Vaccinia Virus (VV). Both *in vitro* with human primary immune cells, and *in vivo* with syngeneic mouse tumor models, PCPV proved to be a very promising vector for immunotherapy. Compared with MVA, PCPV induced a 1000-fold higher expression of IFN-alpha in human PBMCs, whereas SWPV and ORF displayed a lower 10 to 100-fold induction. Other viruses (i.e. VV, CTV, or MYX) did not raise the IFN-alpha level.
When tested for its capacity to trigger the expression of CD86, PCPV was shown to be superior than MVA in primary moDCs. Furthermore, PCPV treatment increased CD86 expression in human *in vitro*-derived $CD163^{+}CD206^{+}$ “M2”-type macrophages, suggesting a shift to an antigen-presenting phenotype. In these cells, PCPV increased significantly the secretion of IL-18, IL-6 and IP-10, signing

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a conversion towards a less suppressive macrophage phenotype.
A recombinant PCPV encoding for the HPV E7 protein was generated to assess the anti-tumor activity and immunogenicity in a syngeneic murine tumor model. Like MVA-E7, PCPV-E7 induced a strong cellular response (ELISPOT on splenocytes, and frequency of antigen-specific short-lived effector cells), but the PCP vector displayed a different cytokine/chemokine profile at the site of injection, with increased levels of pro-immune cytokines including IP-10, IFN-gamma, GM-CSF, IL-18, MIP-1 alpha, MIP-1 beta, IL-12 and IL-6.
When injected intratumorally into fast growing MC-38 tumors, PCPV led to tumor control. Analysis of tumors infiltrates showed that PCPV treatment led to higher levels of neutrophils and decreased the frequency of MHCII^{lo} TAMs.
Our data demonstrate that PCPV might display better properties than current viral vectors, in terms of local response and priming activity, to induce effector T cells and to reshape the tumor infiltration profiles. It has the same capacity as other poxviruses to encode and deliver large genetic payload, which will be useful for designing advanced anti-tumor vaccines.

027

Local radiotherapy and E7-RNA-LPX vaccination activate non-redundant mechanisms and lead to complete tumor rejection of well-established HPV16+ TC-1 tumors

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For head and neck cancers, radiotherapy has been the standard of care for years, using daily low dose irradiation to mediate tumor-rejection. Due to technical improvements regarding precise dose deposition, high dose radiotherapy is increasingly used. Besides inducing growth arrest via DNA damage, high dose radiotherapy mediates immunogenic modulation of the tumor bed, inducing innate and adaptive immune activation.
Therapeutic, RNA-based, cancer vaccines have shown great promise in various pre-clinical and clinical trials driving antigen-specific immune responses. RNA-based cancer vaccines hold the potential to be tailor made, exploiting cancer antigen fingerprints such as E6/E7 antigens in HPV16+ cancers. Anti-tumor responses thereby rely on priming of antigen-specific T-cells as well as promoting their infiltration into the tumor-bed for effector function. Numerous information has been gained about the potency and the potential limitations of E7-RNA-Lipoplex (LPX) vaccines in HPV16+ pre-clinical tumor models. E7-RNA-LPX vaccination drives potent E7-antigen mediated tumor rejection in early TC-1 tumors (Kranz et al. *Nature* 2016). However, in very well-established tumors, E7-RNA-LPX vaccination failed to drive its anti-tumoral effect, raising the necessity for combination therapies. As radiotherapy is the standard of care for head and neck cancers, its combination with E7-RNS-LPX vaccination is an attractive approach to be assessed.
Herein we show a preclinical study treating very advanced HPV16+ TC-1 tumors with E7-RNA-LPX vaccination and different dose radiotherapy. Single E7-RNA-LPX vaccination followed by 14 Gy radiotherapy already lead to complete rejection of 70 mm^3 TC-1 and C3 tumors, generating memory T cells able to prevent TC-1 tumor growth as observed in re-challenge experiments. In order to test the potency of combination-therapy, treatment schedules were performed in more challenging setting, when tumors reached 200 mm^3 size. Weekly E7-RNA-LPX vaccination and two schedules of 14 Gy thereby lead to a rejection of large 200 mm^3 TC-1 tumors. In order to investigate underlying beneficial effects, a study of tumor infiltrating lymphocytes was performed via flow cytometry analysis. We observed potent immune infiltration of E7 specific CD8 T-cells cells upon E7-RNA-LPX vaccination as well as infiltration changes in CD4+ T cells, NK cells and iNOS+ macrophages. In combination-therapy treated mice we additionally observed slowdown of tumor cell proliferation using BrdU-labelling as well as Ki67 tumor cell staining. So far we can speculate that radiotherapy seems to reduce the proliferation of cancer cells, giving E7-specific T-cells the opportunity to attack and eradicate the tumor

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cells.
Taken together, we observed beneficial effects of high dose irradiation and E7-RNA-LPX vaccination in HPV16+ tumor models regarding the induction of tumor-specific CD8+ T cells, tumor growth arrest and modulation of the tumor environment.

028

Standard therapy supplemented with immunogenic cell death therapy during and subsequent multimodal immunotherapy for GBM
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In spite of neurosurgery, radiochemotherapy and maintenance chemotherapy with Temozolomide (TMZm), the prognosis of GBM remains poor. Immunotherapy has shown to improve in some patients the outcome of disease. However, vaccination strategies on its own improved prognosis only in a small fraction of patients. We studied the effect of multimodal immunotherapy as part of first line treatment for patients with primary GBM. Immunogenic cell death (ICD) was induced with Newcastle Disease Virus (NDV) and modulated electrohyperthermia (mEHT) and active specific immunotherapy was performed with autologous mature dendritic cells (DC) loaded with autologous tumor proteins. In a retrospective analysis of 56 patients with primary diagnosis of GBM, we detected 12 adults in whom NDV/mEHT were added at days 8/9/10 during TMZ maintenance (TMZm) cycles, 2 multimodal NDV/mEHT/DC vaccinations were administered after maintenance chemotherapy, and further 3-day NDV/mEHT maintenance immunotherapy were given thereafter. Database was fixed at 01/03/2018. Median age was 60 years (ranging 30-67y). KPI was 85 (ranging 70-100). There was no added toxicity due to ICD during TMZm, or later during multimodal NDV/mEHT/DC vaccinations. Median progression-free survival was 13m. With a median follow up of 18m (range 8-30m), estimated overall survival at 30m was 77% (95%CI: +17, -43). The data suggest that addition of ICD via NDV/mEHT during TMZm might be beneficial in controlling disease progression and improving overall survival. While TMZm only targets dividing tumor cells most when MGMT is methylated, ICD via NDV/mEHT targets both dividing but also non-dividing tumor cells. Finally the active specific immunotherapy induces an antitumoral and anti-viral immune response against NDV/mEHT targeted tumor cells which is maintained during NDV/mEHT maintenance immunotherapy.

029

NKG2A blockade potentiates CD8$^+$ T-cell immunity induced by cancer vaccines
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Cancer vaccination has shown thus far limited clinical efficacy due to multiple suppressive factors in the tumour environment. We now demonstrate that the inhibitory receptor NKG2A constitutes an adaptive resistance mechanism during cancer vaccination by interaction with HLA-E on tumour cells. This immune receptor was preferentially found on tumour-infiltrating natural killer cells and CD8$^+$, but not CD4$^+$, T cells. Expression on CD8$^+$ T cells was found on CD103$^+$ tumour-infiltrating cells and only partly overlapped with other checkpoint receptors. Particularly high frequencies of NKG2A-expressing lymphocytes were detected in tumours with an immune-reactive profile and could be induced by

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therapeutic cancer vaccination. To examine if NKG2A represented an adaptive resistance mechanism during cancer vaccination, we blocked the receptor with a therapeutic antibody and performed genetic knockdown experiments for its ligand Qa-1, the conserved ortholog of HLA-E. In four mouse tumour models, the modest effect of therapeutic vaccines was greatly potentiated by disruption of the NKG2A/Qa-1 axis. NKG2A blockade operated through $CD8^+$ T cells and was even effective in a mouse model refractory to anti-PD-1 therapy. These findings indicate that NKG2A-blocking antibodies might improve clinical responses to therapeutic cancer vaccines .

030

Biomaterial-based scaffolds improve delivery of therapeutic nanoparticle cancer vaccines to dendritic cells

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Synthetic cancer vaccines may boost anti-cancer immune responses by exploiting nanoparticles for co-delivery of tumour antigens and adjuvants to dendritic cells (DCs). These DCs in turn can activate tumor-specific cytotoxic T cells that have the ability to recognize and eliminate tumor cells. However, systemic administration of nanoparticles hampers efficient delivery of cargo and mostly targets DCs residing in immunosuppressive environments such as the tumor microenvironment. Here, we investigate the use of a biomaterial-based scaffold to locally deliver therapeutic nanoparticles, thereby creating a niche that actively recruits DCs. Once DCs enter the scaffolds, they can take up cargo with high efficiency within an immunostimulatory environment. We used alginate, a natural polysaccharide derived from algae, to create macroporous scaffolds that demonstrated unique mechanical behaviour, enabling injection through a conventional needle. We monitored DC viability and migratory behaviour within the scaffolds. Finally, we investigated DC activation in response to nanoparticle-loaded scaffolds and studied antigen-specific T-cell activation. We established that biocompatible alginate scaffolds contain a highly interconnected network, facilitating chemokine-mediated cellular infiltration. Scaffolds functionalized with integrin-binding motifs supported cell adhesion, which enabled DCs to take up PLGA nanoparticles entrapped within scaffold walls. Importantly, this resulted in strong DC activation and acquisition of antigen-specific T-cell-activating capabilities. Our findings demonstrate that carefully designed injectable scaffolds can create a local immunostimulatory niche which facilitates nanoparticle uptake by incoming DCs that subsequently can prime tumor-specific T cells. As these scaffolds can be easily injected into the body, this is a promising approach to recruit DCs to a localized depot of nanoparticles encapsulating synthetic tumor antigens and adjuvants. As such, biomaterial-based scaffolds provide valuable tools to enhance the efficacy of therapeutic vaccination for cancer.

031

Exosomes co-encapsulating antigen and immunoadjuvants act as an effective therapeutic cancer vaccine

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Exosomes are natural nano-vesicles (~30-150nm) secreted from many cells. Recent studies

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suggested that exosomes are excellent vehicles for protein/peptide, gene or short sequences of RNA/DNA delivery. Dendritic cell derived exosomes although used in the immunotherapy of cancers, multiple ligand loading is not possible via cell feeding. Herein, we describe a simple method to externally load ligands within cell-line derived exosomes. Exosomes were first purified from the cell supernatant and loaded with i) cancer antigen ovalbumin (OVA), ii) CpG ODN, and iii) α-GalCer. Therapeutic effect of these exosomes was tested on B16 F10-OVA model. After palpable tumor formation, animals were treated twice either with mixture of 5 µg OVA, 2 µg ODN and 0.06 µg αGC or their loaded counterparts within 30 µg of exosomes. Tumor regression was followed daily by caliper measurement and reported as volume (mm^3) At the end of the experiment tumor infiltrating lymphocytes (TIL) were analyzed by flow cytometry from tumor cell suspensions. Therapeutic effect of the exosome formulation was evaluated by OVA-specific ELISA. Also, splenocytes were treated with MHC-I peptide specific epitope for 72h and $CD8^+$-T-cell specific IFNγ secretion was detected from cell supernatant via ELISA. We found that triple ligand loaded exosome injected group showed significant regression in tumor development compared to PBS or free mixture treated groups (330±120, 1180±440mm^3, 1850±250mm^3 exosome loaded ligands, free ligand mixture and untreated group, respectively) . OVA-specific antibody titers showed development of a Th1-biased anti-OVA immunity after a single injection of exosome formulation (IgG2c/IgG1>1.15±0.3 vs 0.56±0.2; exosome loaded ligand vs free ligand mixture group). Flow cytometry analysis revealed that tumor infiltrating T cells, CD8+ T-cells, CD4+ T-cells, NK cells, NKT-cells, pan and M1-like macrophages were significantly high in lyophilized exosome treated animals compared to PBS and free mixture treated groups. Neutrophilic MDSCs and M2-like macrophages were not different in exosome received animals compared to PBS and free mixture treated groups. Also, we found that when MHC-I peptide epitope was incubated with splenocytes, CD8+ T-cells had significantly higher levels of IFNγ secretion compared to PBS and free mixture treated groups (i.e. 493±120pg/ml vs. 16±4pg/ml; exosome vs. antigen+adjuvant+αGC group). In conclusion, this study suggested that exosomes could be externally loaded with multiple ligands via lyophilization method and these exosomes provide sufficient immune activation and antigen-specific immunity capable of reducing established melanoma in mice.

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Evaluation of immunological changes induced by primary treatment in ovarian cancer patients

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Ovarian cancer is one of the deadliest gynaecological cancers of which widespread metastatic disease is one of the most characteristic features. The current standard primary therapy consists of a combination of radical debulking surgery and platin-based chemotherapy in a neo- or adjuvant setting. However, approximately 80% of the patients relapse. During recurrence, immunotherapies are being considered for treatment application, though without taking into account the immunological changes induced by the conventional therapies. Therefore, we evaluated immune related proteins in serum of ovarian cancer patients during primary treatment.
Serum samples were collected in 14 women with invasive ovarian carcinoma. All of them had a sample taken at diagnosis and at the end of primary treatment (combination of radical debulking surgery and 6 cycles of paclitaxel-carboplatin). Of these 14 patients, 11 received 3 cycles of their chemotherapy in neoadjuvant setting (NACT). The following proteins were measured by Luminex technology: chemokine (C-C motif) ligand 11 (CCL11), CCL24, C-X-C motif chemokine ligand 10 (CXCL10), CCL2, CXCL9, CXCL12, CCL22, CCL5, interleukin 12p70 (IL-12p70), IL-17F, Serum amyloid A (SAA), transforming growth factor beta (TGF-β), IL-10, Galectine-3, matrix metalloproteinase 7 (MMP-7), vascular endothelial growth factor A (VEGF-A), arginase-1, MMP-9, osteopontin (OPN), cluster of differentiation 137/4-1BB (CD137/4-1BB), granulocyte-colony stimulating factor (G-CSF), glucocorticoid-induced tumour necrosis factor receptor-related gene (GITR), granulocyte-macrophage colony-stimulating factor (GM-CSF), granzyme B, interferon gamma (IFN-γ), IL-17A, IL-6, IL-8, FAS ligand and tumour necrosis factor alpha (TNF-α). Statistical analysis was performed using a non-parametric paired Wilcoxon test.
We observed in 11 patients treated with NACT a decrease of TGF-β, arginase-1 and MMP-9 ($p=0.042$, $p=0.0049$ and $p=0.0186$, respectively), compared to diagnosis. In contrast, CCL11 was significantly increased ($p=0.0244$). Overall primary treatment in 14 patients resulted in significant reduction of TGF-β ($p=0.0005$), VEGF-A ($p=0.0107$), CCL22 ($p=0.0203$), arginase-1 ($p=0.0031$), MMP-9 ($p=0.004$), SAA ($p=0.0266$), IL-10 ($p=0.0101$) and CXCL12 ($p=0.0353$), compared to diagnosis.
From this pilot study, it is clear that chemotherapy used in ovarian cancer patients is able to reduce immunosuppression and aggressiveness of the cancer (apart from the increase in CCL11). However, the combination of radical debulking surgery with chemotherapy causes a stronger and more generalised effect. Based on this information, the highly aggressive and immunosuppressive environment that ovarian cancer displays seems to be attenuated and therefore the end of primary therapy seems a good moment to initiate immunotherapy.

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Prognostic relevance of immunological changes during first line treatment in epithelial ovarian carcinoma

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Ovarian cancer is the second most lethal type of gynecological tumor in women with an incidence rate of 12.5 per 100 000. Surgery in combination with platin-based (neo)-adjuvant chemotherapy remains the cornerstone of therapy. The behavior of the immune system during first line treatment is largely unknown. We investigated the influence of primary treatment on the immune system of ovarian cancer on three levels: systemic cellular and humoral immune system and the tumor microenvironment. Tumor biopsies, serum and peripheral blood mononuclear cells were collected prospectively in 41 patients with invasive ovarian cancer. Samples were prelevated at diagnosis, after cytoreductive surgery, after three courses of (neo-)adjuvant chemotherapy, and at the end of their primary treatment. Fluorescent activated cell sorting was applied to detect immunostimulatory cells (T_{helper} cells, $T_{cytotoxic}$ cells, natural killer cells (NK)) and immunosuppressive cells (regulatory T cells (Treg), mMDSC (monocytic myeloid-derived suppressor cells), gMDSC (granulocytic MDSC)). Luminex was used to measure immune related proteins in serum. Immunohistochemistry (IHC) was used to determine $CD8^+$ and $FoxP3^+$ (forkhead bow P3) tumor infiltrating lymphocytes (TIL). IHC showed a significant increase in CD8+ TIL in primary tumor and metastasis after neoadjuvant chemotherapy ($p=0.04$ and $p< 0.01$ respectively), but no correlation with survival. We observed significant changes not only in NK cells, $CD4^+$ T cells, $CD8^+$ T cells and Treg, but also in MDSC subsets in peripheral blood during primary treatment and could correlate the changes in NK cells, CD4+ T cells, Treg, mMDSC and gMDSC to survival. In serum we observed a decrease in CXCL10 (C-X-C motif chemokine ligand 10) after cytoreductive surgery ($p=0.03$) and an increase in CCL2 (chemokine (C-C motif) ligand 2) during adjuvant chemotherapy ($p=0.04$). Conventional first line treatment of ovarian cancer influences the immune system in ovarian cancer, both intratumoral and on a systemic level.

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Peripheral blood NKT-cell frequencies and their PD-1 expression levels identify stage IV melanoma patients with poor overall survival under PD-1/CTLA-4 blockade

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Late-stage melanoma is the deadliest form of skin cancer, which tends to metastasize early on. For patients suffering from this type of cancer, a prognostic indication would be helpful for choosing the most promising therapy. Several studies are searching for new biomarkers that can reliably predict the clinical response and overall survival probability of melanoma patients treated with checkpoint inhibitors. Cancer cells often upregulate ligands for inhibitory receptors on immune cells such as

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cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1). The anti-CTLA-4 antibody ipilimumab and the anti-PD-1 antibodies nivolumab/pembrolizumab are both used in the clinic to treat melanoma. The T-cell population is believed to be the main target of these drugs. Here, we investigated the status of natural killer T-cells (NKT-cells), a small T-cell subset expressing both NK surface receptors and an invariant TCR, in late-stage melanoma. This very heterogeneous population can be divided into two types. In cancer, type I NKT-cells can have anti-tumor functions by the immediate release of Th1 and Th2 cytokines in tissue locations. Type II NKT-cells in contrast may suppress tumor immunity.
In the present study, peripheral NKT-cell phenotypes of 146 stage IV melanoma patients before treatment with nivolumab/ipilimumab or single-agent pembrolizumab were tested for associations with overall survival (OS). NKT-cells were identified by co-expression of CD3 together with CD56 and further subdivided by their CD16 expression. CD16+ NKT-cells are believed to represent mostly type I NKT-cells. Also, the frequency of PD-1-expressing cells was determined.
We observed no associations between frequencies of total peripheral NKT-cells and OS (P=0.652) and only a trend for an association of PD-1 expression levels with clinical outcome (P=0.066). However, a combinatorial model comprising these two factors identified a group of patients with extraordinarily poor OS, characterized by a low frequency of NKT-cells and high PD-1-expression levels (p=0.026). Dividing the NKT-cell population according to their CD16 expression revealed that total CD16+ or CD16- NKT-cell frequencies were not associated with OS (p=0.412). However, high PD-1 expression on CD16- NKT cells was negatively associated with OS (p=0.039).
We conclude that there are significant negative associations between pre-treatment frequencies of NKT cells and clinical outcome in melanoma patients under checkpoint blockade. We suggest that the frequency of NKT-cells, together with their PD-1 expression, represents a novel biomarker to predict poor prognosis under PD-1/CTLA4 blockade. Further investigation of this rarely studied cell compartment is warranted to validate these findings.

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T cell Elispot proficiency panel 2017/2018: Evaluating routine T cell Elispot assays

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Monitoring antigen-specific T cell responses are becoming increasingly important in Immunotherapeutic research and development. Thanks to the harmonization efforts by the CIC and CIMT over many years, and the development of better reagents and protocols, MHC multimer and T cell Elispot assays are now more reliable and accurate assays for monitoring antigen-specific T cell immunity.
Supported by CIMT and CIC, Immudex has conducted a T cell Elispot proficiency panel Winter 2017/2018 to evaluate assay performance in immune monitoring laboratories routinely doing T cell Elispot assays.
39 worldwide participants received two cell each, representing low, medium and high T-cell responses for two predefined peptide pools. A negative control reagent was also included. Participants were asked to use the Direct Human IFNγ Elispot Assay to determine spot count corresponding to each of the predefined peptide pools and the negative control reagent.
After performing the Direct Human IFNγ Elispot Assay, the participants reported back their results for each cell sample as “number of spots per 200.000 viable cells” for each of the predefined peptide pools and negative control reagent.
The data set will be analyzed, and each participating laboratory will receive a report detailing the individual laboratory's performance (in an anonymized format). The anonymized report will become publicly available April 2018, and data will be presented at the 16th CIMT Annual Meeting in Mainz, May 2018.

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MHC class II Dextramers - A new tool for detection of antigen specific CD4+ T-cells

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MHC multimers are important for the detection, analysis and enumeration of antigen specific T-cells in immunological areas of cancer, virus infections, autoimmunity and immunotherapy. While MHC class I multimers are routinely used for detection of antigen specific CD8+ T-cells, the use of MHC class II multimers for detection of antigen specific CD4+ T-cells are less widespread.
The reason for this might be the lower number of antigen specific CD4+ T-cells compared with CD8+ T-cells and that the antigen specific CD4+ T-cells generally carry a TCR with a lower affinity for its cognate ligand, the MHC-peptide complex, than does the antigen specific CD8+ T-cells. Therefore, there is a need for generation of reliable MHC class II Dextramers, that have an increased monomer valency, and thus avidity compared to conventional multimers as tetramers.
Immudex are developing MHC class II Dextramers and have launched the first alleles of HLA class II Dextramers. The MHC class II alleles in development undergo a multitude of tests that ensure their functionality and ability to specifically label antigen specific CD4+ T-cells. We will present data on our MHC class II molecules that shows
1) their ability to be fully loaded with peptides.
2) the peptide binding affinities and characteristics of the MHC class II molecule.
3) the functional MHC class II Dextramer labelling of antigen specific CD4+ T-cells

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High-throughput screening of human tumor-antigen specific CD4 T cells, including neo-antigen reactive cells

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Monitoring of circulating and tissue-infiltrating tumor-associated antigen (TAA) specific CD4 T cells in humans, including neo-antigen specific cells, remains a challenge due to their very low frequency and the absence of reliable tools for their identification. Here we adopted a high-throughput, HLA-independent method for the timely and cost-effective identification, expansion and functional characterization of human tumor-antigen specific CD4 T cells for cancer immunotherapy.
Using this strategy, in healthy individuals, we report frequencies of naive TAA-specific CD4 T cells comparable to those of CD4 T cells specific for infectious agent-derived antigens. Interestingly, we also identified low, but consistent numbers of memory CD4 T cells specific for several TAAs in the circulation of healthy controls. In melanoma patients, before and after peptide-based vaccination, low frequencies of circulating TAA-specific CD4 T cells were detected, with a trend of increased levels post-vaccination. Such TAA-specific CD4 T cell responses were also detected within the tumor tissue by the same approach. By retrieving positive cultures from the libraries, we isolated TAA-specific CD4 T cell clones and performed functional and TCR studies.
Using this screening system, that requires small size input samples, multiple tumor specific CD4 T cell clones are obtained within a 2-week period. This material can be used to develop personalized therapies using tumor-specific T lymphocytes, including neo-antigen specific ones, or gene-

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engineered cells expressing optimal tumor-reactive TCRs isolated from expanded cells.

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Rapid Target Identification for T-Cell Immune Responses with SpotMix™ Peptide Pools
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Enhancing specific immune responsiveness by vaccination is a promising therapeutic approach to infectious diseases and cancer. Its success, however, depends on the identification of suitable target proteins. T-cell target proteins must contain T-cell stimulating peptides providing good epitopes. Whereas algorithms for T-cell epitope prediction have improved significantly in recent years, there is still no reliable method for predicting the most immunogenic targets at the protein level. Stimulation with intact protein or whole protein-spanning, overlapping peptide pools (PepMix™)[1-3] has been successfully used for that purpose, and once a target protein has been identified, individual stimulating peptides can be found by systematic testing, prediction, or a combination of both. However, this approach comes with a significant effort both in terms of labor and cost, in particular if many potential candidate proteins have to be tested. As a result, the number of functionally characterized T-cell-stimulating peptides has increased very slowly in the past 25 years while, thanks to alternative methods such as mass spectrometry, the number of known MHC-associated peptides has increased exponentially.
Based on a method for the highly parallel synthesis of multiple peptides in low quantities, referred to as SPOT synthesis, we have now developed a novel protocol for synthesizing multiple peptides, which includes QC and quantification by LC-MS and permits the production of equimolar pools of SPOT peptides (SpotMix™). Our new protocol is characterized by consistent quality control of thousands of peptides. Comparisons between T-cell stimulation assays (ELISPOT, flow cytometry/intracellular cytokine staining) performed with the classic PepMix™ peptide pools and SpotMix™ pools demonstrate similar performance of these preparations. As SpotMix™ pools typically consist of only 10nmol per peptide and individual peptides are not purified, they are intended for research and discovery only and are, in contrast to PepMix™ peptide pools, unsuitable for ex-vivo T-cell expansions and therapeutic developments. However, we envisage that these novel pools will significantly facilitate T-cell protein target discovery by permitting the synthesis of protein-spanning, overlapping peptide pools for many potential target antigens in parallel. These can be tested empirically and the most promising target proteins can subsequently be selected for more fine-grained epitope mapping.
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039

MemoMAB: Gateway to human antibody repertoires
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Complete access to the human antibody repertoire can be expected to yield valuable insights into

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previously unidentified protective mechanisms active in cancer and other diseases.
The MemoMAB microfluidics-based HT-single cell platform banks and displays authentic antibody repertoires from human donors in recombinant form. MemoMAB processes up to 1mio B cells and directly delivers clonal cell lines expressing recombinant human antibodies. Thus, MemoMAB repertoire biobanks are directly accessible for functional screening of the expressed antibodies enabling antibody discovery from humans and the comparison of antibody repertoires of several donors in parallel.
Using the antibody response as a biomarker, we plan to identify novel immunogenic tumor markers by comparison of responders and non-responders of ICI-therapy.

040

CetuGEX and Cetuximab in recurrent/metastatic squamous cell carcinoma of the head and neck (RM-HNSCC) - p16$^+$ subgroup analysis of the phase 2 RESGEX study

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RM-HNSCC is treated with a combination of cisplatin (P), 5-FU (F), and the EGFR blocking monoclonal antibody (mAb) cetuximab (C) as standard. The mAb CetuGEX (CG) shares the identical EGFR-binding domain with cetuximab, but harbors a defucosylated glycan at the Fc part to increase antibody dependent cellular cytotoxicity (ADCC) by improved binding to the Fcγ receptors IIIa (FcγRIIIa).
240 patients with RM-HNSCC were randomized in 34 European centers, of which 123 received C and 117 CG, in combination with P (100 mg/m^2) and F (4 x 1000 mg/m^2/24hrs) for the first 6 cycles. Initial dose of C was 400mg/m^2, followed by weekly 250 mg/m^2. CG was administered once 990 mg, followed by weekly infusions of 720 mg. After completion of combination treatment, patients received single agent mAb maintenance until disease progression or intolerable toxicity. Stratification factors included FcγRIIIa status, primary tumor site, EGFR pretreatment, and disease stage. Primary endpoint was progression-free survival (PFS) by immune related response criteria (irRC) based on modified RECIST1.1. Secondary endpoints included additional efficacy parameters as well as safety and QoL. Serum CG concentrations and the occurrence of anti-drug antibodies were measured at several time points during the treatment. Furthermore, several genetic and protein biomarkers (EGFR, p16, FcgRIIa, FcgRIIIa) as well as the cellular immune status of the patients were analyzed. Efficacy and safety parameters were assessed in relation to the observed biomarkers, particularly the p16 expression, a surrogate marker for HPV infection.
No advantage of CG over C treatment was observed for the primary endpoint of PFS by irRC [HR 1.003] nor for all other secondary efficacy endpoints and subgroup analyses by stratification factors. In contrast to this, the subgroup of p16$^+$ patients (N=32) is characterized by a significantly longer OS (HR 0.52; 95%-CI 0.29 - 0.86; p=0.009) than that of p16$^-$ patients, probably due to differences in patients baseline cellular immune status. In particular the number of CD4$^+$/ CD8$^+$ T cells and NK cells before treatment is higher for p16$^+$ patients. The clinical benefit for the p16$^+$ patients is only significant under CG (17 patients; HR 0.43; 95%-CI 0.18 - 0.88; p=0.02) but not C treatment (15 patients). For the p16$^+$ subgroup, the number of CD4$^+$ T cells correlates with better PFS and OS.
The RESGEX study is the first head-to-head comparison of an ADCC-optimized to a conventional anti-EGFR mAb. The biomarker assessment, specifically the expression of p16 may help to understand differences between the two mAbs.

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High dimensional analysis of the myeloid cell landscape in human breast cancer metastatic lymph nodes

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In human breast cancer, tumor draining lymph nodes (TDLNs) are the first site of metastasis and lymph node invasion constitutes an important step in disease progression. TDLNs also represent a privilege site where anti-tumor T cell responses are initiated. Migratory dendritic cells can indeed transport tumor associated antigens from the tumor bed to the TDLNs and prime anti-tumor T cell responses. Therefore, TDLNs may play a crucial role for the success of immunotherapies aiming at triggering anti-tumor immune responses. Little is known about the influence of tumor invasion on the immunological status of migratory dendritic cells and how metastasis shapes anti-tumor immune responses initiated in TDLNs. To characterize the heterogeneity and immunological status of myeloid cells in metastatic TDLNs we performed high dimensional analysis (CyTOF and single cell RNA sequencing) of tumor invaded TDLNs from untreated luminal breast cancer patients. We show that: i) TDLNs invasion is associated with a marked increase of $CD14^+$ myeloid cells; and that ii) $CD8^+$ T cells from tumor invaded TDLNs are highly functional compared to exhausted $CD8^+$ T cells present at the tumor bed. In addition, single cell RNA sequencing analysis revealed heterogeneity in the myeloid cell compartment, particularly at the level of the $CD11c^+HLA\text{-}DR^+CD14^{+/-}CD1c^{+/-}$ inflammatory cells. Our results provide a detailed immune cell atlas of the myeloid populations present in metastatic TDLN and suggest a crucial role of TDLNs for the success of immunotherapies.

042

Tumor infiltrating T cells: complete workflows allow faster and improved flow cytometric analysis of syngeneic mouse tumors

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Immunotherapy approaches that engage T cells to attack tumors have proven clinical efficacy and tremendous potential in multiple cancers. Syngeneic mouse tumor models represent the gold standard to develop and analyze effects of immunotherapy, as they possess a fully competent immune repertoire. However, phenotypic and functional analysis of tumor-infiltrating leukocytes (TILs) is technically challenging and labor intensive. The number of tumor infiltrating (TI-) T cells can be very low and small subpopulations might escape analysis as they get lost in the background noise. Importantly, TI-T cells are embedded in a cellular environment where antigen is abundant and surrounding cells express highly immunomodulatory molecules, such that unbiased cell-intrinsic functional characterization is hindered. When working with large cohort sizes, even immunophenotyping of TILs by flow cytometry is time consuming and data processing can be laborious. Therefore, it is fundamental to use effective innovative tools to streamline the workflow and to generate reliable data.
We established complete workflows combining tissue storage, dissociation, T cell isolation and flow cytometric phenotyping. These workflows were validated in 4 different syngeneic mouse tumor models. Tissues were either processed immediately or stored in a solution that was shown to maintain cell viability and phenotype up to 48h after collection (Tissue Storage Solution™). Tumor dissociation was automated and optimized for epitope preservation using a tissue dissociator (gentleMACS™). Phenotypic analysis revealed that optimal enzymatic dissociation was essential for analysis of critical tumor-specific sub-populations, such as PD1hiTim3+Lag3+CD39+CD8+ T cells. We developed new T

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cell-specific enrichment reagents for manual and (semi-) automated magnetic cell sorting, based on MACS® Technology, which enrich for rare TI-T cells by up to 500-fold, while maintaining activation status and phenotype. Importantly, we compared labeling of TILs using conventional hybridoma-derived vs. recombinant antibodies engineered to eliminate Fc receptor-mediated background (REAfinity™). Use of REAfinity antibodies significantly diminished non-specific labeling of cells present in the tumor microenvironment. Finally, flow cytometric analysis was performed using an automated analyzer (MACSQuant X™). This instrument decreased hands-on as well as total acquisition time by facilitating fast and automated sample processing, including sample mixing and absolute cell counting. In conclusion, we have optimized workflows that include standardized processing of tumor samples, newly developed tools for magnetic isolation of TI-T cells and automated flow cytometric analysis. These workflows reduce experimental time and allow more complex experimental setups. We believe using these innovative tools and workflows can significantly increase the quality of data obtained in immuno-oncology research.

043

New and reproducible method for rapid, standardized PBMC preparation and cyroconservation within the scope of clinical trials

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Peripheral blood mononuclear cells (PBMCs) isolated from EDTA-whole blood are widely used in cell-based immunological assays to monitor the effects of immune therapy within the scope of clinical trials. Since cellular immune response assays depend on highly viable and functional PBMCs, the most critical step of those assays is the PBMC isolation and cryopreservation itself. The current standard method for PBMC purification and cryopreservation is the density-gradient centrifugation followed by a temperature-controlled freezing process in media containing serum and DMSO. This technique is time consuming and requires, due to precise whole blood layering over a density medium and careful pipetting of the floating cell layer after centrifugation, well trained personnel. For multicenter clinical trials, it is beneficial to consider a timesaving method, which, in addition, is less operator-dependent and leads to highly viable and functional PBMCs. Here we present the results obtained during verification of PBMC isolation and cryopreservation, were we compared the PBMC isolation by density-gradient centrifugation and a new method using the MACSprep™ PBMC Isolation Kit. Both methods were compared with regard to operator-/ training-dependent variances (2 operators, 4 donors each) in PBMC purity, recovery, and contaminating cells. The PBMC purities obtained by the trained and non-trained operator were almost identical and averaged for the density gradient centrifugation at 86.47% (± 7.18%) and for the MACSprep™ PBMC Isolation Kit at 98.09% (± 1.57%). The non-trained operator achieved more variable results in comparison to the trained operator using the density gradient centrifugation (WBC cell count: - 12%; RBC contamination: + 87%; PLT contamination: + 65%) than using the MACSprep™ Kit (WBC cell count: - 7%; RBC contamination: ± 0% ;PLT contamination: ± 0%). Furthermore, we investigated the impact of different freezing media composition for cryopreservation of isolated PBMC, a cryopreservation medium containing serum and DMSO was compared to StemMACS™ Cryo-Brew. Directly after thawing, MACSprep™ PBMCs, which were cryopreserved with the StemMACS™ Cryo-Brew medium showed a slightly better viability (89.22% ± 2.07%) than density gradient isolated PBMCs, which were cryopreserved with the standard medium (83.85% ± 3.27%). The functionality of T cells within PBMC samples treated differently during isolation and cryopreservation was finally tested with a flow cytometry-based immune monitoring assay, which examines the Interferon gamma (IFNγ) response upon Staphylococcal enterotoxin B (SEB) and Cytomegalovirus (CMV; pp65) or Epstein-Barr virus (EBV; consensus) stimulation. In conclusion, we were able to show, that the MACSprep™ PBMC Isolation Kit in combination with StemMACS™ Cryo-Brew is a time-saving and highly reproducible new method for PBMC isolation and cryopreservation, particularly well-suited for standardized sample preparation in multicenter trials.

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Conventional and immune therapies influence the immune microenvironment of malignant brain tumors

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Conventional therapies and immunotherapies (ITs) have a heterogeneous impact on the immune biology of tumors and this needs to be taken into account to define effective treatment combinations. However, the application of such a concept in high-grade gliomas (HGGs) is hindered by the lack of clear data on this subject. Consequently, the administration of IT after conventional therapy has failed to improve HGG patients' prognosis. The aim of this study was to analyze the influence of local radiotherapy (RT), temozolomide (TMZ) and anti-programmed death-1 (aPD1) treatment on the immune microenvironment of experimental HGGs and to provide the fundamental information required for their effective combination.
CT2A orthotopic HGGs were generated in immunocompetent mice as previously described (Riva et al, ITOC4 2017). From day 12 after tumor inoculation, mice were treated with RT (4Gy, single dose), TMZ (50mg/kg, 4 doses alternate-day) and aPD1 (100ug, 3 doses alternate-day). Magnetic resonance (MRI) was used to measure tumor volumes before starting and after completion of the treatment. On day 18, mice were euthanized, tumor-infiltrating immune cells were isolated via Percoll gradient and characterized with flow cytometry.
Similarly to the clinical setting, RT had the strongest impact on tumor growth even if it did not reach significance (p=0.089) compared to untreated controls (CTRs). The total amount of macrophages (MP) was significantly reduced after each treatment compared to CTRs; however, RT had a stronger effect (p=0.0002) than TMZ (p=0.016) and aPD1 (p=0.045). Both RT and TMZ also decreased M1 MP (p=0.001 and p=0.036, respectively) but only RT significantly reduced M2 MP (p=0.022). There was a trend for an increase of total T cells and of $CD8^+$ T cells after RT. Animals were then stratified in responders (Rs) and non-responders (NRs), according to the fold-increase of tumor volume (on MRI) between day 12 and day 18 (lower or higher than CTRs). Regardless the type of treatment, Rs had a decreased $FoxP3^+$ fraction compared to CTRs (p=0.0227) and to NRs (p=0.0006). Compared to CTRs, Rs also showed a significant decrease in both total MP (p=0.0025) and their M1 and M2 fractions (p=0.0008 and 0.0038, respectively); conversely, NRs showed a non-significant decrease of M1 MP and no change in M2 MP.
This study shows for the first time in detail that each treatment has a peculiar impact on immune cell populations in HGG; interestingly, the strongest immune effect was due to conventional therapies rather than IT. Furthermore, all Rs mice showed a strong reduction of the immune suppressive cells regardless the type of therapy. These findings pave the way for new and synergistic combinatory strategies aiming at driving the immune system to generate a potent anti-tumor response.

045

The blood mMDSC to DC ratio is a sensitive and easy to assess independent predictive factor for epithelial ovarian cancer survival

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Epithelial ovarian cancer (EOC) may cause abnormal blood levels of leukocytes. This paraneoplastic manifestation is associated with a worse response to therapy and shorter survival. To understand the complexity and nature of these leukocytes, we dissected the different populations of myeloid cells and analyzed their relation to clinical outcome. To this end, baseline blood samples of 36 EOC patients treated either with carboplatin/doxorubucin or with gemcitabine were analyzed for different subsets of macrophages, myeloid derived suppressor cells (MDSC) and dendritic cells (DC) using multiparameter flow cytometry as well as functional assays for myeloid cell mediated suppression of antigen-specific T cell reactivity. Healthy donor blood served as control. EOC patients displayed an increase in macrophages, monocytic MDSC (mMDSC) and CD33-CD11b+CD14-CD15- double-negative MDSC (dnCD33-MDSC) and a decrease in the frequency of DC, across all EOC subtypes. A low frequency of DC and high frequencies of macrophages and mMDSC, but not dnCD33-MDSC, were associated with poor overall survival. Patient's macrophages and mMDSC, but not dnCD33-MDSC, were shown to suppress T-cell reactivity in vitro. The mMDSC and DC frequencies were not altered upon treatment. Importantly, the mMDSC to DC ratio was the strongest independent, highly sensitive and specific, predictive factor for survival. This was irrespective of the type of chemotherapy or disease stage and outperformed classical parameters as WHO status or time from last chemotherapy. Thus, the baseline blood mMDSC to DC ratio is a robust, independent and easy to analyze predictive factor for EOC survival, and may assist patient selection for immunotherapy.

046

An improved *in vitro* assay for monitoring antigen-specific T cell responses using synthetic 20mer peptides

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Peptide-based cancer vaccines are one approach to stimulate patients´ immune cells to recognize tumor cells specifically using e.g. neoepitopes or cancer-associated HLA-peptides. These vaccine peptides can be of different length (generally 9-30mers). Long peptides of 20-30mers have the advantage to contain both HLA-class I and -class II potential epitopes. Immune monitoring is of highest importance to assess immunogenicity of peptide vaccines and sensitive assays are needed because of low frequency T cell responses. Current assays have been mostly designed to detect T cell responses using HLA-class I/II peptides of exact length. Here, we present an immune monitoring assay optimized to detect peptide-specific T cell responses against 20mer peptides.
Virus-derived HLA-class I/II epitopes (exact synthetic peptides) were elongated from 9mer or 13-15mer to 20mer peptides according to the original protein sequence. The elongation was implemented either at the N- and C-termini of the exact peptides (class I/II peptides), and in addition at each end only for HLA-class I peptides. Peripheral blood mononuclear cells from healthy volunteers were stimulated with the exact class I/II peptides or the respective elongated peptides for 12 h (*ex vivo* setting) or presensitized for 12 days followed by peptide re-stimulation for 12 h (*in vitro* prestimulation = IVS setting). For the IVS setting, the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) and Hiltonol (Poly-ICLC) to the culture was tested, as well as different peptide concentrations. Specific CD4 and CD8 T cell responses to the various length variants were detected by flow cytometry using intracellular cytokine staining.
The results for the *ex vivo* setting showed specific T cell responses against all tested exact peptides. Although T cell responses were generally also detectable against the elongated peptides, the cytokine level was lower compared to the respective exact peptide. This was especially observed when using N-/C- or C-terminal elongated versions of the class I peptides, whereas N-terminal elongated peptides gave comparable results as for the exact peptide. Unexpectedly, 2 from 3 tested donors showed no T cell response against 1 out of 3 tested HLA-class II peptides, although the response was detectable

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for the respective exact peptide. This lack of reactivity was also observed in the standard IVS setting, however addition of GM-CSF and Hiltonol during the 12-day culture improved recognition of the long peptide. Further experiments in the IVS setting showed that the single addition of Hiltonol is favorable to the detection of both CD4 and CD8 T cell responses.
Overall, we could establish a simple *in vitro* monitoring assay for optimized detection of both CD4 and CD8 T cell responses against long synthetic peptides after IVS.

047

The spleen functions as a window of a systemic response to detect responders to immune checkpoint inhibitor treatment in murine progressed solid carcinomas by [^{18}F]FDG positron emission tomography (PET)

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Immunotherapy using immune checkpoint inhibitors (CIT) is a powerful tool to treat progressed solid carcinomas. Nevertheless, the majority of the patients does not benefit from the treatments or is suffering from severe side effects. Thus, early identification of non-responders is essential to enable changes to novel CIT combinations or different treatment regimes.
Recently, we reported on the development of a novel therapeutic approach in mice with progressed insular cell carcinomas using repeatedly a combination of two checkpoint inhibitors, anti-PDL-1 and anti-LAG-3 mAbs (PDL1+LAG3) and weekly transfers of tumor antigen-specific T cells (TA-TH1) following an initial immune cell depletion mediated by 2Gy whole body radiation. Importantly, only the combination (2Gy+TH1+PDL1+LAG3) was highly efficient. Using non invasive [^{18}F]FDG-PET scans, we have detected an increasing glucose metabolism during the treatment course in the spleens of mice responding to the combined treatment. Here, we aimed to identify immune cell populations responsible for the splenic metabolic changes using flow cytometry and immunohistochemistry (IHC). Expecting that TA-TH1 and endogenous T cells are responsible for the enhanced splenic glucose metabolism, we examined the distribution and activation state of the splenic T cell populations: While the relative amount of CD3$^+$ T cells was reduced to 7.4±0.66% following PDL1+LAG3 treatment (combined with or without TA-TH1) compared to isotype mAbs treatment (ISO, 10.35±2.1%), they yielded a higher activation state (CD69$^+$). Notably, adoptively transferred TA-TH1 cells replaced 30% of the endogenous CD4$^+$ T cell population. Histopathological and IHC analysis revealed intense atrophy of T- and B cell regions (CD3, B220 stainings) but increased granulopoesis (ASDCL staining) while ISO treated mice (harboring a lower glucose metabolism) revealed a normal appearance of the white pulp and no granulopoesis. Considering the reduced amount of splenic T cells our results indicate that the enhanced splenic glucose metabolism might be rather linked to the enhanced splenic granulopoesis.
To conclude, this is a first report that connects an enhanced splenic glucose metabolism, demonstrating a systemic immune response, to the efficacy of a combined T cell-based checkpoint inhibitor immunotherapy. Thus, non invasive *in vivo* imaging using [^{18}F]FDG-PET scans of the spleen functions as a window to non invasively identify non-responders to the treatment before irreversible cancer progression. In parallel, we focus on clinical studies in tumor patients with ICP-blockade to approve our hypothesis.

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Metabolic profiling of secondary lymphatic organs using 18F-fluorodeoxyglucose (FDG) Positron Emission Tomography (PET)/CT together with eosinophil blood count, S100 and LDH levels is applicable to stratify patients with metastatic melanomas responding to checkpoint inhibitor immunotherapy (CIT)

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There is growing evidence that successful cancer immunotherapy requires a systemic anti tumor response involving secondary lymphoid organs and a complex interplay between different immune cell populations. Unfortunately, the exact mechanisms and dynamics are not discovered yet due to difficulties in the *in vivo* assessment of immune responses. Inspired by preclinical studies on experimental murine tumor models, we retrospectively studied in vivo 18F-FDG PET/CT and routine clinical data of patients with metastatic melanomas in order to gain deeper insights into the systemic immune response induced by CIT. Since CIT do not improve overall survival in a significant number of patients we focused on differences in immune response patterns between responders and non-responders.

In preclinical experiments we were able to differentiate between effective and non-effective immunotherapies by analyzing the 18F-FDG-uptake in the spleen. In total, data from 38 patients with metastatic melanoma with 18F-FDG-PET/CT scans before and start of therapy with CTLA-4 or PD-1 Ab were available. For further analysis, patients with PET studies within 50d before and 100d after therapy start were identified (13 responder: 2x nivolumab; 6x pembrolizumab; 4x ipilimumab 1x ipilimumab+nivolumab; 10 non-responder: 1x nivolumab; 5x pembrolizumab; 4x ipilimumab). Regions of interest (ROI) in the spleen were defined in the CT images and copied to the coregistered PET for semiquantitative analysis. Blood parameters were obtained from the clinical routine laboratory.

We observed no significant differences in relative neutrophil and lymphocyte counts between responders and non-responders but elevated eosinophils counts in responders before the start of CIT. Baseline 18F-FDG-PET/CT-scans before CIT revealed no differences in the spleen 18F-FDG-uptake (SUVmean: 1.7±0.1 vs.1.8±0.1) between responders and non-responders. After onset of CIT routine tumor markers S100 and LDH increased in non- responders but decreased in responders: 243±131% vs. -11±22% (S100) and 46±26% vs. -13±13% (LDH). Surprisingly, we observed an elevation of the relative eosinophils counts by 129±75% in non responders but a decline by -20±24% in responders whereas the relative lymphocyte counts declined in either responders (-18±10%) and non responders (-15±7%). In line with our preclinical studies, the follow up 18F-FDG-PET/CT-scans provided a tendency towards a higher 18F-FDG uptake in the spleens of responders when compared to the baseline 18F-FDG-PET/CT-scans (+3.5±9% vs. -1.4±4%). Interestingly, the SUVpeak (the 1cm3 with the highest 18F-FDG uptake) negatively correlated with the decrease in relative eosinophil counts.

Thus, non invasive in vivo 18F-FDG-PET/CT imaging of the glucose metabolism in secondary lymphatic organs combined with tumor marker-/LDH-values and the eosinophil blood count might represent a powerful tool to predict an immune response for early identification of responders and non-responders.

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Development of an imperacer (Immuno-PCR) assay combining broad assay range and excellent sensitivity to support development of a TNF-receptor agonist antibody drug

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Background: Biologics such as therapeutic antibody drugs are often administered in wide concentration ranges for dose-response evaluation. Safety and potency considerations frequently demand low dosing in clinical trials and therefore very sensitive ligand-binding assays (LBA) for PK analyses. In contrast, significantly higher dosing has to be applied in pre-clinical safety assessment for fast cleared drugs needing high upper limits of quantification. Here we describe an ultra sensitive Imperacer assay combining a broad assay range of greater than4 logs with excellent sensitivity in the low pg/ml range, to support development of a TNF-receptor agonist antibody drug.
Methods: Immuno-PCR (IPCR) based Imperacer LBA technology in sandwich-format, using surface immobilized capture reagent in combination with marker-DNA tagged detection conjugate for qPCR signal generation.
Results: As part of a technology evaluation study, an Imperacer assay to quantify a therapeutic antibody for cancer treatment was developed with a preliminary assay range of 2 - 32,768 pg/ml and an LLOQ aiming at 6 pg/ml with good accuracy and precision. Selectivity testing at 6 - 10 pg/ml was tested in 10 human serum samples from healthy volunteers to define LLOQ level. Prozone (hook) effect was found starting around 1 µg/ml, however dilution linearity confirmed that concentrations up to 10 µg/ml can be diluted into the assay range with acceptable recovery. Higher concentrations were not tested at this point. This assay as developed for a feasibility approach has the potential to significantly reduce time and effort in LBA PK sample testing support for this Biologic, as the sponsor uses two different platforms to measure the range of concentrations in serum. The assay is not yet fully optimized and can be adapted towards further study requirements prior to method validation.
Conclusion: Exponential signal amplification driven combination of excellent sensitivity and broad assay range on the Imperacer platform can significantly reduce bioanalytical sample testing time and effort during all phases in the development of Biologics.

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Ultra sensitive PK bioanalysis in support of the development of a bi-specific immune-recruiting biotherapeutic

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Background: Bispecific antibody formats recruiting T cells as effector cells (e.g. tetravalent bispecific antibodies) are in development as biotherapeutic drugs for a variety of indications, primarily in cancer therapy. Their excellent specificity and selectivity in combination with their ability to recruit immune-effector cells to tumor tissue makes them very attractive candidates in fighting cancer.
However, T cell-based therapies have been associated with TOX profiles (e.g. potential cytokine release syndrome) which need to be carefully managed and based on their high potency, initial dosing is very low in clinical trials. In consequence, the expected drug concentrations in PK profiling are well below the technical limitations of conventional ELISA-type ligand-binding assay support. Thus, novel assays are required to overcome these limitations.
Method: Imperacer Immuno-PCR assay development and bioanalytical-method-validation in support of clinical trials.
Results: The PK assay for tetravalent bispecific antibodies was developed on Chimera's IPCR platform Imperacer with +4 log assay range from 5 pg/ml - 75,000 pg/ml and fully validated according to bioanalytical method validation guidance documents. A special focus lay on matrix adaptation via Chimera's AnySource sample dilution technology. GCP bioanalytical analyses to support clinical

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studies is ongoing with excellent assay performance in different hematologic patient sub-populations.
Conclusion: Here we present assay development and method validation of an ultra-sensitive PK assay for GCP regulated bioanalytical sample testing support of a T cell recruiting bispecific tetravalent antibody in development for the treatment of hematological malignancies. The provided combination of broad assay range and high sensitivity is a powerful tool in PK analysis of low dosed drug concentrations.

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Bioanalytical PK support for Immunotherapeutics: case studies
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Background: Immunotherapeutic concepts play an increasingly important role in modern drug development. The most common class of immune-modulatory drug candidates are antibodies or recombinant constructs thereof, which can pose mayor difficulties in dosing. The therapeutic dosing regime of immunotherapeutics may vary significantly from fairly low to rather high dosing, depending on safety, binding targets, potency, clearance rate, physiological effects and other considerations. Hence, for optimal trial support, not merely sensitivity but likewise expansive assay range of the supporting method is key. However, most ligand-binding assay (LBA) technologies either lack sufficient sensitivity or have considerable restrains in continuous dynamic range.
Here we present state-of-the-art bioanalytical approaches to overcome these limitations in GxP regulated study support, discussing several case studies from the development of immunotherapeutic compounds. Related aspects, e.g. LBA support of studies with limited sample volume availability, as e.g. typical in Ophthalmology are discussed as well.
Method: Ultra sensitive, Imperacer Immuno-PCR (IPCR) technology in sandwich-format, using surface immobilized capture reagent in combination with marker-DNA tagged detection conjugate for exponential qPCR signal amplification and read out.
Results:
Case Study 1) Immunomodulator Agonist Antibody Drug:
An IPCR immunossay, using two anti-ideotypic antibodies was developed for quantification of an immunomodulatory antibody drug candidate in human serum samples for phase I oncology trial support.
The assay demonstrates a calibration curve range from 2 - 32,800 pg/ml with excellent acceptance criteria. Due to prozone effect observed at 1 µg/ml, ULOQ can be extended to approx. 100,000 pg/ml if required.
Case Study 2) Bi-Specific Antibody Drug:
Using the recombinant binding target of one arm as capture and an anti-idiotypic antibody as detector, this PK assay supports the development of a bi-specific antibody for treatment of hematological malignancies with approx. 1 pg/ml - 6,000 pg/ml assay range. Dilution linearity was confirmed up to 360 µg/ml, allowing an even extended assay range. Assay specificity was confirmed by sample parallelism of neat, 1:1,000 & 1:100,000 diluted pre-clinical samples.
Case Study 3) Diabody Drug:
For clinical PK sample testing support of a bi-specific diabody under development as oncology therapy, an immunossay ranging from 100 fg/ml - 800 pg/ml was developed. In-development analysis of residual samples from a previous clinical trial was performed. Consistently, all pre-dose samples were found to be negative (BLQ), while dosing could be monitored down to sub pg/ml concentrations for this biologic
Conclusion: Due to exponential signal amplification driven combination of sensitivity, broad assay rage and tolerance for sample dilution, the Imperacer IPCR platform is well suited for LBA sample testing support of low dosed biologics.

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Finally, empty HLA class I proteins! A disulfide-stabilized variant of HLA-A*02:01

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The peptide ligand is critical for maintaining the conformational stability of HLA class I proteins. This has obstructed the parallel production of multiple recombinant class I-peptide complexes and necessitated various peptide exchange technologies. We now present and characterize a novel variant of HLA-A*02:01 that has a disulfide bond linking the alpha-1 and the alpha-2 helices in the vicinity of the F pocket region. In silico, molecular dynamics simulations show that this new disulfide bond dynamically stabilizes the peptide binding site just like a full-length peptide. In vitro, the disulfide-stabilized (DS) variant can be folded to yield peptide-empty HLA class I molecules that are stable in solution, freeze-thaw compatible, and that bind peptide with rapid kinetics. The disulfide bond is far away from the known footprints of T cell receptors (TCRs), suggesting that TCR recognition of the DS variant will be close, or identical, to that of the HLA-A*02:01 wild type. We will discuss applications of the DS variants in HLA multimer staining and in T cell isolation and activation.

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Empty MHC class I molecules: An alternate, convenient, and improved detection tool for antigen specific T cells

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Major histocompatibility complex (MHC) class I multimers have been widely used to identify antigen specific T cells for immune monitoring, epitope discovery, and T cell isolation. Bottleneck to cover the breadth of antigens specific to >10000 peptide-HLAs is the peptide ligand dependent stability of these proteins, which thus compels high-affinity peptide dependent *in vitro* folding of each MHC protein and use an exchange technology to investigate antigens of interest.
We now investigate empty peptide-receptive MHC class I molecule, an HLA-A*02:01 variant which is stabilized by a disulfide bond to link the alpha-1 and alpha-2 helices outside the binding sites of both peptide and T cell receptor(TCR), to detect antigen specific T cells. We directly incubated antigenic peptide ligands of viral origin (specific to HLA-A*02:01) with these empty and stable HLA-A*02:01 molecules, made fluorescence labelled tetramers and used them to detect previously known T cell responses in healthy donor peripheral blood mononuclear cells. In all tested samples, disulfide-stabilized HLA-A*02:01 tetramers detected T cell with same specificity as wild type MHC tetramers. Since, mainstream cancer immunotherapy requires detection of cancer specific antigens for both clinical and immunomonitoring applications, and these easy to load MHC molecules would be a definite prospective to utilize given the breadth T cell epitopes, we extended our analysis to detect cancer specific antigens in melanoma tumor infiltrating lymphocytes and identified T cells with previously known specificity using wild type molecules. Interestingly the disulfide-stabilized HLA-A*02:01 multimers provide consistently a better staining index for detection of antigen specific T cells, conveying a substantial advantage when using these reagents for the analyses of low avidity T cell interaction, such as autoantigen recognition.
To evaluate if the disulfide linkage have an impact on TCR recognition of peptide-MHC complexes, we determined and compared TCR fingerprints of T cell clones specific to a given peptide-MHC complex using both wild type and the disulfide-stabilized HLA-A*02:01 multimers. No differences were

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observed in the TCR incitation profile between the disulfide optimized and the wild type MHC class I Putting together, disulfide-stabilized empty HLA class I proteins are potentially powerful for T cell staining and isolation, and for epitope discovery.

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Immunomonitoring for personalized cancer immunotherapies via *ex vivo* combinatorial workflow and concurrent high-resolution Class I & II ICS

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Within the last decade Cancer Immunotherapy has gained remarkable interest with many different therapeutic approaches showing the potential to revolutionize treatment of cancer. Numerous clinical trials investigating the safety and therapeutic potential of checkpoint inhibitors, adoptive cell transfer therapies, bispecifics, as well as combinatorial approaches are under way. This strongly increases the need for reliable assays, capable to draw a detailed picture of the underlying immune responses, which determine the outcome of cancer immunotherapy. Therefore, we have developed in the view of the limitations of patients' blood two different flow cytometry-based assays, which allow an unbiased monitoring of immune responses in great detail and with outstanding sensitivity: First, an ex vivo assay combining Class I 2D-multimer (2DMM) staining with Class II intracellular cytokine staining (ICS) assay. The Class I 2DMM staining allows us, to follow the fate of cancer-specific CD8+ T cells with a potential frequency of one cell in up to one million CD8 T cells. At the same time the Class II ICS enables the determination of cytokine production by CD4+ T cells, ex vivo and hence in completely unbiased manner. Second, we developed a pan ICS assay including a single in vitro sensitization step to analyze a broad array of cytokines produced by CD4+ T helper (TH) cells and CD8+ CTLs in parallel. These two assays have been developed as part of the EU-funded GAPVAC project to address immune responses in a phase I trial applying actively personalized peptide vaccines to glioblastoma patients. The exceptional sensitivity and flexibility of these assays could be demonstrated even in this highly demanding and variable clinical setting, demonstrating strong induction of peptide-specific CD8+ T cells, as well as detection of a broad range of different cytokine responses and T cell phenotypes induced upon peptide vaccination.
Hence, altogether the combined ex vivo assay is capable to generate immunomonitoring data with outstanding sensitivity and reliability, while the “pan ICS” approach covers a broad range of cytokines allowing to analyze the phenotype and function of CD4 and CD8 T cells. The approach thus optimally exploits the limited number of available patient material for generation of highly relevant immune response data.

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Scalable end-to-end immune repertoire analysis: the ImmunoGenomiX Platform

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High-throughput sequencing of the immune repertoire is increasingly used for monitoring tumor biomarkers and evaluating the performance of immunotherapeutics for cancers. However, most of the available tools are not designed to track and investigate the dynamic nature of B and T cell receptor repertoires across multiple time points or between different patient cohorts in a clinical context.
We introduce the ImmunoGenomiX Platform - a scalable end-to-end immune repertoire analysis platform that is currently under development. This platform allows quantitative characterization of

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immune repertoires from sequenced B or T cell receptors. The modular structure of the ImmunoGenomiX Platform and its integration with data management systems allows users to manage and process large clinical studies, stratify patients, filter clonotypes and interactively explore analysis results.
Using TCR sequencing data, we show how the intuitive graphical user interface of the ImmunoGenomiX Platform makes analysis and interpretation of repertoire data accessible to clinicians and biologists alike, without the need for computational expertise.

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Tumor epitope detection in monocytes (EDIM) as a marker for antitumoral activity of Newcastle disease virus infusion and modulated electrohyperthermia

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Active specific tumor vaccination with autologous dendritic cells is an emerging treatment strategy for several cancer types. Feasibility and lack of toxicity is demonstrated in numerous clinical trials. In order to improve the potency for the induction of effective tumor control, combination approaches are studied, including oncolytic virotherapy and modulated electrohyperthermia (mEHT), both inducers of immunogenic cell death (ICD). Patients (n=24) were treated with daily infusions of Newcastle Disease Virus (NDV) and with mEHT as part of multimodal immunotherapy. Blood was followed with flow cytometry on a daily basis for change of two tumor epitopes in monocytes using the EDIM test: the DNaseX/Apo10 proteine epitope, a marker of abnormal apoptosis and proliferation, and Transketolase-like 1 (TKTL1), a marker for anaerobic glucose metabolism (Warburg effect). Patients were in median 54 years (range 3-79). Cancer categories were neuro-oncology (n=11), urologic (n=2), gynecologic (n=5), digestive (n=5) and pulmonary (n=1) oncology. For all cancer types we found a significant increase in the score for Apo10, TKTL1 and the sum of both from the first till the sixth treatment. In 9 patients, we were able to follow the same variables during a second week of treatment, scheduled 3 weeks after the first. The scores were returned to the basic value, but again increased significantly upon daily NDV/mEHT treatment. The data demonstrate that NDV/mEHT target tumor cells, and induce ICD with release of Apo10 and TKTL-1, taken up by circulating monocytes and detected in EDIM test.

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Peripheral blood γδ T-cell frequencies correlate with overall survival of stage IV melanoma patients under PD-1 blockade

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γδ T-cells represent a numerically minor subset of 1-10% of the peripheral T-cell compartment with a major role in defense against multiple viral, microbial and nonmicrobial challenges. Unlike the majority of T-cells, γδ T-cells bind their ligands in an MHC-independent manner and mediate unique functions, best described as bridging adaptive and innate immunity. Vδ1 T-cells are associated with viral infections, especially with immunosurveillance against human Cytomegalovirus (CMV). Vδ2 Tcells are

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notably involved in immunity against bacterial infections.
Recently, interest has resurged in the possibility of using γδ T-cells in cancer immunotherapy, because these cells can kill melanoma cells in vitro, and possess regulatory capabilities. Thus, γδ T-cells could potentially influence the efficacy of immunotherapies. We showed previously that frequencies of peripheral γδ T-cells are associated with outcome in melanoma patients. Correlations of γδ T-cells with outcome were consistent in cohorts with various treatment backgrounds and in patients under CTLA-4 inhibition.
Here, using polychromatic flow cytometry, we characterized peripheral blood γδ Tcells and their differentiation subsets in a cohort of 84 stage IV patients prior to PD-1 blockade. Constellations of co-stimulatory and co-inhibitory receptors on γδ T-cell subsets were profiled in a subgroup of this cohort via mass cytometry. Confirming previous studies, peripheral frequencies of Vδ1+ γδ T-cells were negatively associated with patients´ overall survival (OS ; p=0.045). Because a latent infection with CMV markedly alters the distribution of peripheral T-cell subsets, including γδ Tcells, and could thus act as a confounding factor, we stratified the patients according to CMV status. We observed no association of OS with CMV seropositivity. However, a superior OS was identified in the sub-group of CMV-seropositive patients with low Vδ1+ T-cell frequencies relative to either CMV-seronegatives or those with high Vδ1+T-cell frequencies (P=0.027). Associations between frequencies of Vδ1+ γδ Tcells and OS have now been observed in 3 independent studies. We therefore conclude that this subset represents a novel biomarker in melanoma and that CMV seropositivity confers a survival benefit.

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Disappearance of NY-ESO-1 and Melan-A-specific T-cells from peripheral blood is associated with clinical response in melanoma patients undergoing anti-PD-1 therapy

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Increasing numbers of clinical trials administering anti-PD-1 antibodies emphasize the requirement for predictive biomarkers of clinical response and overall survival (OS) for this treatment regimen. Lactate dehydrogenase is thus far the only well-accepted biomarker for malignant melanoma. We have previously shown that the presence of functional NY-ESO-1 or Melan-A-specific T-cells was significantly associated with prolonged survival in late-stage melanoma patients not undergoing immune checkpoint therapy. In order to determine whether the presence of tumor-associated antigen (TAA)-specific T-cells is capable of predicting clinical response or OS in anti-PD-1-treated patients as well, we assessed T-cell responses against NY-ESO-1 and Melan-A in n=100 stage IV melanoma patients before and during treatment.
We detected functional antigen-specific T-cells recognizing NY-ESO-1 and Melan-A after 12 days of in vitro expansion with overlapping protein-spanning peptides at two time points (baseline and 4 weeks after the first injection). After restimulation, the readout assay was intracellular cytokine staining (ICS) measuring 4 functional markers simultaneously (IFN-γ, TNF, IL-17 and CD107a). This allowed us to analyze the phenotype and the function of antigen-specific T-cells at the single-cell level. Detection of functional influenza-specific T-cells (MP1 antigen) served as a positive control. We additionally determined the frequencies of various myeloid (e.g. MDSC) and lymphoid (e.g. regulatory T-cell) immune populations in the same samples.
We observed that unlike in patients not treated with anti-PD-1, neither the presence of NY-ESO-1- nor Melan-A-reactive T-cells before treatment was associated with prolonged survival. However, comparing both measured time points, we discerned a loss of NY-ESO-1 or Melan-A-specific T-cells during treatment in 79% of all clinical responders (complete response and partial response according

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to RECIST criteria) ($p < 0.0001$; Fisher´s exact test). The frequency of influenza-specific T-cells was not affected. Importantly, we did observed no such a disappearance of NY-ESO-1- or Melan-A-specific T-cells in any of the patients who progressed. Consequently, the one year survival rate was 92% in patients with a loss of TAA-specific T-cells under treatment, compared to 64% in patients without such a loss.
Taken together, our data confirm an important role for TAA-specific T-cells also for successful checkpoint-immunotherapy trials. The finding that NY-ESO-1- or Melan-A-specific T-cells have to be present before anti-PD-1 therapy, but must disappear from blood for a good clinical response (possibly due to migration in the tumor), also supports the use of these shared TAA in future vaccination or ACT trials prior to the application of anti-PD-1 antibodies.

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Monitoring of T cell infiltration and positioning in colorectal cancer liver metastases using a human tissue-based ex vivo cell migration analysis model

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High densities of tumor infiltrating lymphocytes (TIL) in patients with colorectal cancer liver metastases (CRC-LM) have been positively connected with therapy responses. However, effector T cells are mainly observed in the invasive margin of patients and not in the tumor epithelium. Such T cell distribution could hinder an effective anti-tumor immune response. Little is known about lymphocyte infiltration and positioning in tumor microenvironments (TME). Especially detailed therapy effects on TIL are difficult to detect in patients. In addition, the strong differences between mice and human regarding the immune system and the TME restrict reliable monitoring of therapy effects on the human immune response in mouse models. For these reasons, a human CRC-LM tissue-based ex vivo cell migration analysis model was established to detect and localize exogenous infiltrating as well as endogenous lymphocytes in authentic human TMEs. In brief, autologous TIL were isolated from tumor adjacent liver tissue, labeled with a fluorescent dye and returned to the tissues, which were cultured for a particular time period. By further use of immunostaining, imaging and cell quantification, we found that the remigration of exogenous TIL into the models was significantly associated with endogenous TIL quantities. Moreover, in respect of localization, the distribution patterns of exogenous TIL were found similar to those of endogenous TIL with highest densities in the invasive margins of patient samples. Further cytokine and chemokine quantification revealed significant associations of exogenous TIL infiltration with concentrations of the chemokines MIG, IP10 and CCL5. The results clearly show that lymphocyte infiltration into CRC-LM always occurs in the same manner. Microenvironmental factors such as MIG, IP10 and CCL5 seem to guide and position lymphocytes into the invasive margin preventing contact with the tumor epithelium. The successful reproduction of lymphocyte infiltration and distribution highlights the functionality and reliability of the human tissue-based cell migration analysis model. Especially the similarity of TIL positioning in the ex-vivo and in-vivo settings emphasizes the use of the model for studying therapy effects on TIL distribution in the TME of CRC-LM patients.

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CD47 expression predicts efficacy of macrophage-mediated phagocytosis of tumor cells

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Most human cancers express CD47 to escape macrophage surveillance. Reinstating macrophage functionality in clearing tumor cells targeting CD47 has been an important strategy in immuno-oncology therapy. We profiled a large panel of tumor cell lines of both solid and blood cancers that are frequently used in oncology drug discovery, and in MiXeno™ animal studies at Crown Bioscience, for CD47. Despite CD47 being reported as an ubiquitously expressed immune-escape marker in cancer cells, expression levels varied among the different tumor cell lines following examination by flow

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cytometry. It is of interest that many tumor cells do not in fact present CD47 on their cell surface, despite gene expression data (e.g. RNAseq data) suggesting that they do.
To establish a robust *in vitro* assay platform to support anti-CD47 drug research, we developed a macrophage differentiation system followed by M1/M2 activation. Activated macrophages were co-cultured with target tumor cells, with labeling for flow cytometry analysis. In the presence of anti-CD47 antibody, a steady phagocytosis effect was observed with target tumor cells expressing high levels of CD47 markers. To confirm these findings, we constructed isogenic cell models from the same parental cells in which the CD47 gene is overexpressed. The isogenic systems were subjected to CD47 biology studies, and can serve as positive controls in phagocytosis assays.

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The oncometabolite R-2-hydroxyglutarate suppresses T cell immunity in IDH1-mutant gliomas

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The oncometabolite R-2-hydroxyglutarate (R-2-HG), accumulating in IDH1-mutant gliomas to high millimolar levels, promotes gliomagenesis via glioma cell intrinsic DNA and histone methylation. However, R-2-HG is exported from glioma cells into the extracellular space and can be detected in the body fluids of patients with IDH1-mutant cancers. While T cell infiltration is reduced in IDH1-mutant gliomas, the functional consequences of extracellular R-2-HG accumulation for glioma-infiltrating immune cells and glioma immune-surveillance are unclear. Based on the hypothesis that R-2-HG suppresses antiglioma immunity via direct paracrine effects, we evaluated the impact of R-2-HG on T cell function using (i) expression, metabolic profiling and functional assays of primary human and murine T cells, (ii) RNA-sequencing of freshly isolated human glioma TILs and immunohistochemistry in a large glioma cohort, and (iii) preclinical IDH1-mutant specific T cell transfer, IDH1-mutant peptide vaccination, and checkpoint inhibition in syngeneic murine intracranial and flank tumor models as well as novel astrocyte-specific IDH1R132H knock-in models. R-2-HG produced by mutant IDH1 is transported into T cells via sodium-dependent solute carriers. Intracellularly accumulating R-2-HG constrains mitochondrial respiration, which leads to suppression of ATP-dependent T cell receptor (TCR) signaling. As a consequence hallmarks of both R-2-HG-treated primary T cells as well as T cells infiltrating IDH1-mutant gliomas, are reduced calcium signaling and nuclear factor of activated T cells (NFAT) transcriptional activity. ATP depletion alsoleads to activation of AMP-activated protein kinase (AMPK) and thus inhibition of ornithine decarboxylase (ODC) 1, which results in impaired polyamine synthesis. Interestingly, R-2-HG also directly inhibits ODC1 activity. Due to these effects, T cell activity and effector functions are impaired by R-2-HG both in vitro and in human WHO grade II and III glioma tissues, as evidenced by reduced proliferation, cytokine production and PD-1 expression. In vivo, genetic or pharmacologic inhibition of mutant IDH1 enzymatic activity enhances T cell-mediated antitumor immune responses against experimental syngeneic IDH1-mutant flank and intracranial tumors, which either occur spontaneously or are induced by IDH1R132H-specific vaccination or adoptive T cell transfer or by immune checkpoint blockade. These data attribute a novel, non-tumor cell-autonomous role to an oncometabolite in shaping the tumor immune

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microenvironment. Inhibitors of mutant IDH, which block R-2-HG production and are currently in early clinical development, may represent a new class of small molecule checkpoint inhibitors to be combined with PD-1 blocking antibodies or vaccines in IDH1-mutant cancers.

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Tumoral MHC class II expression in gliomas

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Neoantigens are rare in tumors with low mutational load such as gliomas. Evidence has been gained that these tumor-specific antigens are mainly presented on major histocompatibility complexes (MHC) class II (MHCII) molecules eliciting a tumor-specific T helper cell response. Albeit the fact that T helper cells are required for tumor control in preclinical tumor models and direct induction of tumor cell senescence has been suggested, underlying molecular mechanisms still remain incoherent. For MHCII-restricted vaccines, cytotoxic T cells are still required for antitumor activity e.g. by MHC class I-restricted cross-presentation or target-independent intermolecular antigen spread by reprogramming of the immunosuppressive tumor microenvironment.
Here we evaluate stromal and tumoral MHCII expression and its functionality in gliomas, a disease in which targetable MHC class I-restricted neoantigens are rare.
Immunohistochemistry and immunofluorescence of human glioma tissue were used to identify tumoral, endothelial, and microglial MHCII expression and to enumerate T cell infiltrates. To evaluate an MHCII expression-associated immune signature, expression and CIBERSORT analyses from the TCGA dataset were performed. MHC ligandome analyses of an MHCII+ glioma cell line and human glioma tissues were used to evaluate functionality of MHCII *in vitro* and *ex vivo*. Functional *in vitro* co-culture assays using an HLA-DR-matched tetanus toxoid (TT) epitope-overexpressing glioma cell line and buffy coat-derived and *in vitro*-expanded TT-reactive T cells from healthy donors were used to evaluate direct target recognition by T helper cells.
MHCII was expressed in the majority of gliomas and associated with an increased infiltration of T cells and a T helper cell signature. In 10% of the analyzed glioma tissues, tumoral MHCII expression was detected. Ligandome analyses revealed functionality of MHCII molecules on glioma cells. In *in vitro* co-culture assays, TT-reactive T helper cells specifically produced IFN-g when co-cultured with target expressing, MHCII+ glioma cells and co-stimulation was provided.
Tumoral MHCII expression is present in gliomas. T helper cells are in principle capable to form functional immunological synapses with glioma cells. Further experiments are required to evaluate the predictive value of tumoral MHCII expression in the context of MHCII-restricted neoepitopic vaccines.

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Role of 14F7 monoclonal antibody immunoreactivity against N-glycolyl GM3 ganglioside in colon cancer prognostic

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Introduction: Given the limited impact of conventional factors in colon cancer (CC), it is necessary to identify new prognostic biomarkers. N-glycolyl neuraminic acid is not usually detected in human normal tissues, however N-glycolyl containing gangliosides has been found in a variety of human malignancies suggesting its possible role on the oncogenic process, tumor growth and progression and becoming in attractive targets for cancer immunotherapy. The expression of N-glycolyl GM3 ganglioside (NeuGcGM3) have been previously reported in different tumors using 14F7 monoclonal antibody (Mab), a murine IgG1 highly specific against the variant N-glycolylated of GM3 ganglioside produced by the Center of Molecular Immunology, Havana, Cuba.
Aim: To assess the prognostic role of 14F7 Mab immune-reactivity, against N-Glycolyl GM3 ganglioside, in patients with colon cancer and to evaluate the relationship between its expression and clinic-pathological features.
Methods: Paraffin embedded specimens were retrospectively collected from 50 patients with CC operated between 2004 and 2008. 14F7 Mab staining was determined by immunohistochemistry technique according to proportion of stained cells and intensity of 14F7 Mab reactivity. The proportion of stained cells was graded on a scale of 0-3 (0, no staining; 1, 1-50%; 2, 51-75%; and 3, 76-100%). The staining intensity was graded on a scale of 0-3; 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Subsequently, an immunoreactive scoring was obtained by multiplying the two previously mentioned parameters and its relation with survival and clinic-pathologic features was evaluated.
Results: The reactivity of 14F7 Mab was detected in all cases. Most cases had high level of immune-staining (70%) that showed statistical correlation with TNM stage (= 0.025). In univariate survival analysis, level of 14F7 Mab immune-reactivity (= 0.0078), TNM Stage (= 0.0007) and lympho-vascular invasion (0.027) were significant prognostic factors for overall survival. Among these variables, level of 14F7 Mab immune-reactivity (HR = 0.268; 95% CI 0.078-0.920; = 0.036) and TNM stage (HR = 0.249; 95% CI 0.066-0.932; = 0.039) were independent prognostic factors on multivariate analysis.
Conclusions: This study is the first approach on the prognostic significance of 14F7 Mab immune-reactivity in patients with colon adenocarcinoma and this assessment could be used to estimate the prognosis of CC patients, although further studies will be required to validate these findings. Furthermore, the role of NeuGcGM3 in tumor biology and its differential expression in tumor cells support its potential use as target for immunotherapy passive and active.

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Human recombinant Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) produced by *Lactococcus lactis* bacteria acts synergistically with 5-Fluorouracil and Metformin to induce apoptosis in colon cancer cells *in vitro*

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Introduction: TRAIL induces apoptosis in cancer but not normal cells, however, the use of soluble TRAIL in the clinic is mostly restricted by its short biological half-life after systemic administration. Another problem concerning potential TRAIL-based anticancer therapy is, in some cases, cancer cell resistance to TRAIL-induced apoptosis. It has been shown that metformin, the most widely used anti-diabetic drug, acts synergistically with TRAIL and restores/enhances sensitivity of cancer cells to TRAIL-induced apoptosis. The objective of this project is to assess the effect of human soluble TRAIL (hsTRAIL), produced by nonpathogenic *Lactococcus lactis* bacteria, on the survival of tumor cells of the HCT116 and SW480 human colon cancer cell lines *in vitro* and *in vivo*. A long-term TRAIL delivery/secretion to the tumor microenvironment, controlled by these bacteria, may serve locally as an

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effective tumor cell elimination mechanism. In this project, the potential synergistic effects of hsTRAIL in combination with 5-fluorouracil (5-FU), the chemotherapeutic used in colon cancer treatment, and metformin (MetF) were examined.
Materials and methods: Recombinant plasmid pNZ-hsTRAIL harbouring codon-optimized hsTRAIL-cDNA was constructed and transformed *via* electroporation into *L.lactis* NZ9000 host cells. Synthesis and secretion of hsTRAIL by *L.lactis* clones was determined by PCR, ELISA and Western blot. Screening *in vitro* of *L.lactis*-derived hsTRAIL antitumor activity alone and in combination with 5-FU and MetF added to the cultures of HCT116 and SW480 human colon cancer cells were examined using MTS assay. Induction of apoptosis in colon cancer cells by hsTRAIL alone or synergisticaly with cytostatics was determined by flow cytometry (FITC-Annexin V and propidium iodine-staining).
Results: Western blot analysis and ELISA confirmed the efficient production of hsTRAIL by the recombinant *L.lactis* clone. *L.lactis*-derived hsTRAIL exhibited apoptotic activity on both human colon cancer cell lines and acted synergistically with 5-FU and MetF. Furthermore, *L.lactis* hsTRAIL-secreting bacteria reduced the number of HCT116 colon cancer cells *in vitro*.
Conclusions: *Lactococcus lactis* bacteria produce biologically active hsTRAIL, which acts synergistically with 5-FU and MetF, thus, eliminating human colon cancer cells *in vitro*. More studies are required to assess the safety and efficiency of *L.lactis*(hsTRAIL+) clone as a vector for TRAIL delivery *in vivo*.
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Protumoral effects of TLR7 in lung tumors

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TLR7 agonists are currently under investigations for their ability to enhance anti-tumor immune responses. However, in some tumor models, these agonists also stimulate malignant cells, which can express high levels of TLR7, a receptor for single-stranded RNA [1].
We have demonstrated that stimulation of lung tumor cell lines expressing TLR7 with synthetic TLR7 agonists led to upregulation of the antiapoptotic protein Bcl-2, tumor cell survival and chemoresistance [2].
In Non-Small-Cell Lung Carcinoma (NSCLC) cohorts of patients, we have observed high expression of TLR7 on malignant cells in 70% of patients, which conferred poor clinical outcome and was strongly associated with resistance to chemotherapy [3]. This pro-tumoral effect of TLR7 has been validated in murine models in which the injection of TLR7 agonists in NOD/SCID mice, in C57BL/6 wild-type or in TLR7-deficient mice grafted with lung adenocarcinoma tumor cells led to increased tumor progression, increased lung metastasis, and resistance to chemotherapy. On the contrary, we demonstrated that TLR7 antagonist injection led to antitumoral effect.
Additionally, TLR7 stimulation modified the tumor immune microenvironment resulted in a significant increase of Myeloid-Derived Suppressor Cells (MDSCs). Depletion experiments of MDSCs indicated that these cells are involved in the pro-tumoral effect induced upon TLR7 stimulation. Finally, we have demonstrated that the pro-tumoral effect of TLR7 stimulation, mediated by the MDSC recruitment, was independent of TLR7 stimulation on immune cells. Our results reveal the mechanism by which TLR7 stimulation induce the pro-tumoral effect in mice, and open the way to the development of novel cancer therapeutics including TLR7 inhibitors, for NSCLC patients.
This results demonstrate the important role of TLR7 in lung tumor progression. Knowing that natural ligands of TLR7 are ssRNA, we suppose that this effect could be linked to viral infections or RNA released in the tumor microenvironment.

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Durable response to PD-1 blockade in a patient with mismatch repair deficient advanced biliary tract cancer

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Clinical trials with immune checkpoint inhibitors have identified a subset of patients throughout different tumor types that develop a potent and durable anti-tumor immune response. Most of these tumors are characterized by a high mutational burden associated with immunogenic mutation-induced neoantigens. Mismatch repair deficieny and microsatellite instability (MSI) lead to the generation of a large number of neoantigens, triggering an adaptive immune response. However, while MSI is actively discussed as a predictive biomarker for response to PD-1 blockade, the extent and duration of treatment response vary significantly between MSI-H patients. Here, we report on a 28-year old woman diagnosed with extrahepatic cholangiocarcinoma who showed strong and durable response to the immune checkpoint inhibitor pembrolizumab, although treatment was initiated at an advanced stage of disease. The patient's tumor displayed DNA mismatch repair deficiency and microsatellite instability (MSI), but lacked other features commonly discussed as predictors of response towards checkpoint blockade, such as PD-L1 expression or dense infiltration with cytotoxic T cells. Notably, high levels of HLA class I and II antigen expression were detected in the tumor, suggesting a potential causal relation between functionality of the tumor's antigen presentation machinery and the success of immune checkpoint blockade. We therefore aim at characterizing the molecular determinants predictive of PD-1 treatment response in MSI-H patients and determining mechanisms of immune escape at treatment failure.

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Dominant oncolysis in a syngeneic lung cancer model treated with LCMV-GP - pseudotyped vesicular stomatitis virus

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It is now well established that the therapeutic effect of oncolytic virotherapy is largely based on a two-pronged approach - the direct action of tumor-selective infection, viral replication, and cell killing and the associated activation of innate and adaptive immune responses with the potential of long-lasting tumor remission. We used a chimeric vesicular stomatitis virus pseudotyped with LCMV glycoprotein (VSV-GP), which we have previously reported to have both a rapid lytic cycle and a broad tumor tropism; to address the direct oncolytic effects and the role of antitumor immune induction in the syngeneic mouse lung cancer model LLC1. *In vitro*, VSV-GP was found to efficiently infect and lyse LLC1 cancer cell-lines. Exogenously applied interferon type 1, however, conferred resistance to VSV-GP action demonstrating a dependence of the oncolytic effect on defects in the IFN response of

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cancer cells. Using a matched pair of LLC1 wildtype and interferon receptor knockout tumors (LLC1-IFNAR$^{-/-}$) *in vivo*, interferon insensitivity of cancer cells correlated with prolonged intratumoral viral replication and improved therapeutic outcome. Luciferase imaging revealed successful tumor-to-tumor spread of viral progeny in bilateral tumor models. Histological analysis confirmed widespread and rapid infection and cell killing within the tumor. To assess the contribution of adaptive immunity, particularly CD8^{+} T cells we compared the efficacy of VSV-GP therapy in treating LLC1-IFNAR$^{-/-}$ tumors in the presence or absence of cytotoxic T-cells using immune-incompetent nu/nu hosts or CD8-depleting antibody regimen. In syngeneic mice, the absence of CD8^{+} T-cells was associated with slightly enhanced lysis of LLC1-IFNAR$^{-/-}$ tumors by VSV-GP. In addition, when re-challenged with the same tumors, surviving mice showed little protection suggesting lack of effective anti-tumor immunity. Together, these studies present a case for a lytic-dominant treatment effect of VSV-GP in a syngeneic mouse lung cancer model. Future studies will focus on improving the balance between facilitating viral oncolysis and enhancing anti-tumor immunity.

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A 3D image-based quantification of tumor-immune cell interactions in the presence of immuno-modulators

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Introduction: Unleashing the full potential of immunotherapies and maintain durable clinical responses requires understanding of cellular mechanisms that govern anti-tumor immune responses. However, the progress in this direction is hampered by a lack of appropriate pre-clinical testing models that are both clinically relevant and suitable for routine screening of drug candidates. Therefore, we developed a robust in vitro assay that allows image based high-throughput analysis of 3D cultures. Here, immune cells are co-cultured with cancer cells in a 3D environment which recapitulates the tumor micro-environment and its complex cellular interactions. That allows for functional read-outs, such as infiltration of immune cells into the organoids and their killing. These effects are visualized and measured in a relevant spatial context, which enables a better understanding of the immune-modulatory profile of different immunotherapies.
Material and Method: Tumoroids generated from cancer cell lines or autologous colon organoids from normal and tumor tissue from several patients were cultured in vitro in a 3D environment. Immune cells derived from healthy donor PBMCs or CAR-T cells with and without activation were added and their infiltration into organoids and subsequent killing was visualized using high-content microscopy. Quantification of immune cell effects was achieved with morphometric analysis with OMiner^{TM} software.
Results and Discussion: 3D image data analysis enabled the discrimination of immune-tumor cell interactions and revealed a higher immune cell infiltration and tumoroid killing upon activation. In addition we observed differential responses to immune-modulation between normal and tumor tissues, which highlighted patient related differences. These results elucidate the effect of immune cell targeting normal colon tissue and discriminate immune-tumour cell interactions depending on activation status of T cells. The 3D environment, both for the cell culture and image analysis, allows for measurement of spatially resolved information, not accessible by monolayer cultures or biochemical assays.
Conclusion: The 3D assay presented here allows visualization and measurement of effects of immunotherapies on cells that engage in a more physiologically relevant spatial setting than when culturing them in traditional 2D cultures. Using image-based analysis, immune-tumor interactions can be clearly dissected and analyzed. Using both healthy and tumor tissue from the same patient allows for better prediction of clinical outcome versus non-specific cytotoxic reactions. This offers immunotherapy drug developers a new and innovative platform to select the most promising candidates and understand their mechanism of action, which ultimately translate to better clinical performance.

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Targeting Stat3 oncogene addiction together with anti PD-1 antibodies as an effective combination for cancer immunotherapy

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Stat3 is constitutively activated in diverse cancers and acts as a critical mediator of tumor immune evasion. Previously, we described in murine breast cancer (BC) models, that blockade of Stat3 activation induces senescence and that immunization of mice with irradiated Stat3-blocked BC cells inhibits tumor growth. Although senescent cells are growth arrested, they remain metabolically active and develop a senescence-associated secretory phenotype (SASP) that can have pro as well as anti-tumorigenic effects. Our objectives were to study the senescence mechanism induced by Stat3 blockade and to characterize and develop an immunotherapy (IT) based on the secretome of Stat3-blocked cells. Here we report that Stat3 knockdown induced senescence markers in diverse tumor types including BC, colon cancer and melanoma. Stat3 inhibition only induced senescence in cells addicted to Stat3, meaning that they depend on Stat3 activation to maintain its malignant phenotype. Moreover, we observed that the SASP from Stat3-blocked cells increased the proliferation of T lymphocytes and the number of IFNɣ producing CD4+T cells *in vitro*. In addition, this secretome inhibited proliferation of endothelial cells (HUVEC) when compared to control secretome. These data suggest that the SASP from Stat3-blocked cells can promote an anti-tumor activity. To test this hypothesis *in vivo*, we designed an immunization protocol based on the administration of the SASP from Stat3-blocked cells. We used supernatant (SN) from 4T1 (BC) or B16-OVA (melanoma) cells transfected with Control siRNA (SN-Control) or Stat3 siRNA (SN-Stat3) as a slow depot adjuvant of a cellular vaccine based on irradiated wild-type tumor cells. Therapeutic IT with SN-Stat3 in mice bearing 4T1 or B16-OVA tumors decreased tumor growth compared with SN-Control. In 4T1 tumors, we also observed a decrease in pulmonary metastasis and an increase in activated NK cells and CD4+T cells in spleen vs. SN-Control. Addition of an anti-PD-1 antibody, that itself has no impact on B16-OVA growth, enhanced the antitumor activity of the SN-Stat3 IT, delays B16-OVA tumor growth and increases tumor infiltration and IFNɣ-producing antigen specific CD8+T lymphocytes. Next, we characterized the components of SN-Stat3. With a multiplex cytokine array we detected an increase in CD4+T and NK cells-attracting chemokines and IFNɣ-induced cytokines (IP-10, RANTES, TNFα and IL-15) compared to SN-Control. Then we performed a SILAC-based quantitative proteomics to decode the secreted proteins. We found enrichment in proteins involved in cell adhesion, metabolic and immune system processes. Our work suggests that Stat3 inhibition in several tumors can activate a senescence-associated antitumor immunity. These results demonstrate that cytokines and proteins released by Stat3 blockade from tumor cells can be used to formulate an effective adjuvant to enhance the antitumor effect of anti-PD-1 antibodies.

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Epigenetic modifications upregulate PD-1, CTLA-4, TIM-3 and LAG-3 immune checkpoint genes in breast tumor microenvironment

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High expression of immune checkpoints in tumor microenvironment plays a significant role in inhibiting anti-tumor immunity, and it is associated with poor prognosis and cancer progression. The major

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epigenetic modifications in both DNA and histone could be involved in the upregulation of these immune checkpoints in cancer. We first assessed the expression level of different immune checkpoint genes in breast tissues, and found that PD-1, CTLA-4, TIM-3 and LAG-3 are significantly upregulated in breast tumor tissues, compared with breast normal tissues. To investigate the epigenetic modifications behind this upregulation, we checked both DNA and histone methylations of these immune checkpoints. Interestingly, we found that the CpG islands in the promoter region of PD-1, CTLA-4 and TIM-3 were significantly hypomethylated in tumor tissues compared with normal tissues, but not LAG-3. The demethylation percentage of immune checkpoint genes was highest in CTLA-4 followed by PD-1 then TIM-3 and finally LAG-3. Moreover, bindings of trimethyl histone H3 lysine 9 (H3K9me3) and histone H3 lysine 27 (H3K27me3) were reduced in the promoter loci of PD-1, CTLA-4, TIM-3 and LAG3 in tumor tissues. These results show that both DNA and histone epigenetic modifications are involved in the upregulation of PD-1, CTLA-4 and TIM-3 in breast tumor tissue. Additionally, only histone methylation was involved with the upregulation of LAG-3 in breast tumor microenvironment. Taken together, our data reveal one of the underlying mechanisms involved in the upregulation of multiple immune checkpoints in breast tumor microenvironment, and these epigenetic modifications could be potential diagnostic/prognostic biomarkers and/or therapeutic targets in breast cancer.

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CD200 mimetic PEG-M49 and doxorubicin co-treatment increases CD4CD8 double positive cells in CD200R1 knockout mice and the levels correlate with its anti tumoral effects

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CD4CD8 double positive cells increases following viral infections and they are antigen-specific with high effector potential. Increased levels of CD4CD8 double positive cells were also detected in malignancy and autoimmune diseases. Although the role of CD4CD8 double positive cells in anti-tumoral immunity is not know, their role in clearance of viral infection suggest that they have cytotoxic activity against tumor cells. CD200, a cell surface glycoprotein, inhibits autoimmune diseases by acting through CD200 receptor 1 (CD200R1) and has immune suppressive effects. Despite its immune suppressive effects, CD200 overexpression in the host decreased metastasis of highly aggressive 4THM murine breast carcinoma suggesting that CD200R1 and CD200 may have distinct effects. We here examined the effect of a CD200 mimetic PEG-M49 and Pegylated liposomal doxorubicin (PEG-Dox) on breast cancer metastasis and the level of CD4CD8 double positive cells in the presence and absence of CD200R1.
4THM cells were injected into the mammary-pads of three groups of Balb/c mice: wild type (WT), CD200 knockout (CD200ko) and CD200R1 knockout (CD200R1ko). Five days after injection of tumor cells, mice were injected with Peg-Dox (ip total dose was 17,5mg/kg) and PEG-M49, or PEG-cApt (control) (iv). Changes in tumor growth, lung metastasis, and CD4 and CD8 positive cells in draining lymph nodes, spleen and tumor tissues were determined.
PEG-M49 and Peg-Dox co-treatment suppressed primary tumor growth more effectively in CD200R1ko mice than WT and CD200ko mice. Tumor growth and metastasis was increased in CD200ko mice demonstrating that CD200 has anti-metastasis effects. PEG-M49 and Peg-Dox co-treatment markedly suppressed tumor growth and metastasis but the effect was most prominent in CD200R1ko mice. In correlation, co-treatment also increased tumor infiltrating CD8+ and CD4CD8 double positive cells (app. 6 fold) in CD200R1ko mice but not in wild type. In draining lymph nodes of CD200R1ko mice, there was a major shift such that almost all CD8+ cells were also CD4+ both under treated and untreated conditions. Differently co-treatment increased double positive cells in lymph nodes of all groups, though to a different extent. Co-treatment increased CD4+ CD8- cells and did not altered the levels of CD4-CD8+ cells suggesting that double positive cells were not originated from single positive cells. Somewhat similar changes were observed in spleens such that co-treatment

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markedly increased double positive cells in wild type and Cd200ko mice without negatively altering the number of single positive cells. These results demonstrate that increasing CD200R1 activity inhibits the level of circulating double positive cells which might have an important therapeutic implication for autoimmune diseases. However the opposite seems to be true for aggressive carcinomas such that inhibition of CD200R1 together with enhancing CD200 activity may provide more potent anti-metastatic activity.

072

The effects of TRPV1 agonists on immune response to metastatic breast cancer as well as on tumor growth and metastasis

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TRPV1 receptors are activated by capsaicin, the compound in chili pepper responsible for its “hot” taste. TRPV1 receptors were mostly expressed on afferent sensory neurons; hence majority of the studies has focused on neuronal functions of the TRPV1. TRPV1 receptors are also expressed in immune cells as well as cancer cells. Activation of TRPV1 channels of cancer cells decreases cell proliferation suggesting anti-tumoral effects of TRPV1 channels. Because TRPV1 receptors are also present in immune cells and nerve fibers, they are likely to alter tumor growth and metastasis by regulating cancer immune response. Hence we here evaluated the effects of TRPV1 receptor agonists on immune response to metastatic breast cancer as well as on tumor growth and metastasis.
Balb-c mice were injected with brain metastatic subset of 4T1 murine breast cancer cells (4TBM) and treated with two different TRPV1 antagonists; Capsaicin and Olvanil. Capsaicin was given either a week before or one week after injection of tumor cells as a single dose (25mg/kg) into the fat pad of neck region. This was done to provide slow release of highly fat-soluble capsaicin from fat tissue. Olvanil (0,1 mg/kg and 1mg/kg) was given 5 days after injection of 4TBM cells. Changes in tumor growth and metastasis as well as cytokine secretion of stimulated mix leukocyte culture (MLC) obtained from spleen and draining lymph nodes of tumor bearing animals.
Capsaicin inhibited lung metastasis markedly when given a week before injection of the 4TBM cells. Capsaicin given a week after injection was not tolerated well and 4 out of seven animals died immediately after injection. Capsaicin pre-treatment markedly inhibited TNF-a and IL-6 release from MLC in response to LPS and irradiated 4TBM cells challenge. Olvanil on the other hand did not markedly altered tumor growth and at low doses (0,1 mg/kg) increased lung metastasis. Olvanil at higher dose (1mg/kg) decreased LPS-induced IL-6 release without altering metastasis. These results demonstrated that different TRPV1 receptor agonists may alter tumor growth and metastasis differently which might be related to pharmacodynamic and pharmacokinetic differences of the agonists. Because olvanil at 1mg/kg dose inhibited IL-6 release, which is an important mediator of metastasis and tumor aggressiveness, we are currently examining the effects of olvanil at higher doses.

073

VSV-GP therapy is effective in HNSCC though direct tumor lysis and stimulation of an anti-tumor immune response

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VSV-GP combines the tumour cell killing efficacy of Vesicular Stomatitis Virus (VSV) with an enhanced safety profile, making it an excellent candidate for clinical development as treatment option for advanced cancers. VSV-GP mediated cell lysis releases tumour derived antigens, which in combination with the viral components, such as the viral RNA genome unleash a strong anti-tumour immune response. We have evaluated VSV-GP for the treatment of head and neck squamous cell carcinomas (HNSCC). HNSCC is one of the most common cancers worldwide with half a million new cases each year. The 5-year survival rate is around 50%; risk factors include alcohol, smoking and HPV infection.
We first tested VSV-GP using a human xenograft mouse tumor model of HNSCC. Treatment resulted in a solid response in most animals with some lasting remissions. To confirm the ability of VSV-GP to infect and kill human tumor cells in a more translational setting we infected primary human HNSCC tissue slices obtained from fresh biopsies of HNSCC patients. Using this approach, we confirmed that VSV-GP can indeed infect and kill primary, patient derived tumors.
We next tested the VSV-GP treatment in a syngeneic mouse model of HNSCC to confirm that besides tumor cell lysis VSV-GP also activates anti-tumor immunity. SCC VII cells are derived from a HNSCC, which was chemically induced in C3H mice. Upon intratumoral (i.t.) treatment VSV-GP replication is restricted by innate anti-viral immunity, in this model. Nevertheless, i.t. treatment resulted in approx. 50% remissions and long term survival of treated mice. We hypothesised that these effects are due to a virus-induced anti-tumor immune response and confirmed this by expression and immunohistological analysis of treated and control tumors, where we observed enhanced immune infiltration in regressing tumors. To further strengthen our claims, we successfully re-challenged cured animals, demonstrating that they were protected from tumor growth, in contrast to naïve, age matched control animals that readily developed tumors.
In conclusion, we demonstrated that in addition to direct tumor cell killing VSV-GP treatment leads to a strong as well as durable anti-tumor immune response and immunological memory formation. This makes VSV-GP a potent oncolytic virus to treat HNSCC and a good candidate for combination therapy with other immune therapies.

074

Adipose tissue drives accumulation and activation of mast cells in human cancer specimens

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In a multitude of retrospective studies, the impact of mast cell numbers and localization within the tumour microenvironment on patient outcome has been analysed - however present knowledge did not yield an unambiguous consensus with clinical data yet, mainly emphasizing that the presence of mast cells contributes to a complex functional network in the immune landscape and that their effects are not only depending on clinical aspects like tumour entity, TNM and grading, but also on their spatial distribution and activation status. Preliminary results, indicating an accumulation of tryptase$^+$ cells around adipocyte-rich areas led to a further examination of their localization and effector status in comparison between fat cell-rich and non-fat cell-containing tumour tissues.
Immunohistochemical stainings for common mast cell, T- and B-cell markers and quantitative image analysis have been performed on slides of ovarian and colorectal cancer specimens. We could show so far that tryptase$^+$ cells, as well as CD3$^+$T- and CD19$^+$ B cells in ovarian and colorectal cancer are not only highly expanded in the presence of fat, but reside predominantly in a 500µm-wide boundary around adipocytes. Mass spectrometry was employed to characterize the activational phenotype of mast cells in ovarian cancer by histamine release and quantification of 50 immune-related cytokines was performed in multiplex assays on tissue lysates derived from the same patients. Comparing non-

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fat with fat-containing ovarian carcinomas we found differences in mast cell attracting and -activating molecules, which were shown to be elevated in the latter and potentially drive an activated phenotype - an effect that was not detectable in mast cell-containing tumours lacking fat tissue.
The yet limited spectrum of spatial cell analysis will be broadened to a range of other cancer entities, preferentially those arising in an adipocyte-rich environment, including mammary and pancreatic cancer.
Overall, these results indicate a potential role of mast cells in fat mediated pro-tumourigenic inflammation and could yield novel insights leading to a higher clinical effort to target this cell type in diverse human cancers. In order to further unravel their role in adipose tissue inflammation we aim to (I) culture mast cells in adipose tissue supernatant and analyse cytokine profiles, (II) characterize the spatial expression of cytokines and chemokines and (III) examine the interaction between mast cells and B-cells.

075

IL27Rα deficiency alters stromal cell architecture and delays tumor growth in a polyoma middle T oncoprotein-driven breast cancer model

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IL-27 is known to have dual roles in cancer biology, either enhancing anti-tumor immunity or promoting tumor-supporting inflammation. In the present study we specifically investigated the role of IL-27 on stromal cells during tumor development in a *polyoma middle T oncoprotein* (PyMT) driven breast cancer model. PyMT breast cancer cells were transplanted into mammary glands of IL27Rα wildtype and knockout (KO) mice, respectively. Tumor growth onset and progression were markedly delayed when tumors grew in IL27Rα KO hosts. When looking at tumor immune cell composition, we observed a remarkable reduction in the overall immune cell infiltrate including all major immune cell subsets, with the exception of macrophages. Tumor histology revealed reduced proliferation, and greater hypoxic and necrotic areas within the tumors of KO mice. Moreover, mRNA expression of target genes of the hypoxic transcription factor Hif1α was increased in tumors of IL27Rα KO mice, indicating disturbed perfusion. Injection of fluorescence-labelled BSA and immunohistochemical stainings indicated malformation of vessels, which could explain the reduced immune infiltrate as well as the enhanced hypoxia and necrosis in tumors growing in IL27Rα KO mice. We conclude that in our model, IL-27 acts pro-tumorigenic by enabling proper angiogenesis and hence ensuring the supply of oxygen and nutrients necessary for tumor growth. These findings need to be considered when envisioning IL-27 as a target in tumor immunotherapy.

076

Induction of myeloid-derived suppressor cells via tumor-derived extracellular vesicles in malignant melanoma

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Malignant melanoma (MM) accounts for almost 80% of all skin tumors deaths. The accumulation of highly immunosuppressive myeloid-derived suppressor cells (MDSCs), which arise from immature myeloid cells (IMC) in the bone marrow, plays a significant role in the immunosuppression and in the

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resistance to immunotherapy in MM. It was shown that melanoma cells could generate MDSC by secreting extracellular vesicles (EVs), which have been proved to be essential in intercellular communication. In addition, EVs promote the progression, invasion and metastasis of cancer. However, the mechanisms of MDSC generation and activation by EVs from MM remain to be explored.
We have shown that the treatment of IMCs with endotoxin-free EVs induced the secretion of inflammatory cytokines such as IL-1β, IL-6, IL-10, TNF-α and COX2. In addition, a strong upregulation of PD-L1 was detected. By studying myD88-and TLR2/4/7-knockout mice, we found that these alterations were mediated by the stimulation of the NF-kB activation mainly by the TLR4 signaling pathway. Moreover, TLR4 signaling was shown to be largely triggered by heat-shock proteins, which are sorted into EVs by cells undergoing stress. By inhibiting heat-shock proteins at the transcriptional level, we could completely abrogate the EV-mediated PD-L1 upregulation on IMC. Functional assays showed that EV-treated IMC become immunosuppressive. They could inhibit the proliferation of CD8+ T cells and reduce the production of interferon-γ. Interestingly, the impact of EV-treated IMC on T cell functions was mainly due to the PD-L1 upregulation. To validate the importance of these results *in vivo*, we injected EVs into C57BL/6 wild type and TLR-4 knockout mice. Educating wild type mice with EVs led to the accelerated tumor growth, whereas $TLR4^{-/-}$ mice did not display under these conditions any modulation of tumor growth.
Our data suggest that tumor-derived EVs convert IMC into functionally active MDSC via upregulation of PD-L1 expression mediated by the TLR4 signaling.

077

The safety, tolerability, and preliminary clinical activity of the CXCR4 inhibitor X4P-001 and nivolumab in renal cell carcinoma patients that are refractory to nivolumab monotherapy

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Background: X4P-001 is an oral, selective, allosteric inhibitor of the chemokine receptor CXCR4 in development for treatment of patients (pts) with advanced renal cell carcinoma (RCC). In a prior clinical study of advanced melanoma pts, X4P-001 showed preliminary evidence of enhanced immune cell infiltration, including increases in activated CD8+ T cells and interferon gamma signatures. Nivolumab, an anti-PD-1 checkpoint inhibitor currently approved for RCC, enhances antitumor CD8+ T cell responses but does not alter immune cell trafficking. It is hypothesized that X4P-001 in combination with an anti-PD-1 will enhance immune cell infiltration of tumors in pts that are refractory to checkpoint inhibitors alone, leading to improved clinical outcomes.
Methods: In this ongoing, open-label study, the safety, tolerability, and preliminary clinical activity of X4P-001 in combination with nivolumab was assessed in pts with advanced RCC. All study pts were required to be receiving current nivolumab therapy for advanced RCC with a best response of stable disease (SD) or progressive disease (PD) by RECIST v1.1 criteria. Patients were administered 400 mg QD oral X4P-001 while continuing on 240 mg nivolumab therapy by intravenous infusion every 2 weeks.
Results: As of the data cutoff date of 7 February 2018, 9 pts enrolled in the study. The median age was 65 years (range: 49-77) with 8 males and 1 female. Patients had received a median of 4 prior lines of therapy (range: 1-7), and all pts had a baseline ECOG performance status of 0 or 1. Three pts are receiving ongoing combination therapy, and 2 pts have discontinued from the study due to treatment-related adverse events (AEs). Of the 8 clinically evaluable pts, there was one partial response (PR), 6 pts with SD, and 1 pt with PD. All 4 pts with PD on nivolumab at study enrollment had SD with the addition of X4P-001 treatment (median duration: 33 weeks; range: 16-42). Of the 4

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pts with SD on nivolumab at study enrollment, 1 had a PR. Combination treatment-related AEs (≥15%) were diarrhea and nasal congestion (4 pts each); dry eye (3 pts); and alanine aminotransferase increase, conjunctival hyperemia, dyspepsia, fatigue, and rash pruritic (2 pts each). One pt each (11%) experienced grade ≥3 treatment-related AEs of increased lipase, mucosal inflammation, and rash. **Conclusions:** Combination therapy with 400 mg QD oral X4P-001 and standard doses of nivolumab is well-tolerated in pts with advanced RCC. Interim efficacy assessments provide preliminary evidence of clinical activity with the combination and suggest that X4P-001 could potentially augment tumor responses in pts who are refractory to checkpoint inhibitor therapy alone.

078

Single-cell transcriptome analysis unveils a heterogeneous and dynamic landscape of tumor infiltrating CD8 T cells

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The response of naive CD8 T cells to acute infections results in the generation of defined yet heterogeneous pools of effector and memory cells. In contrast, the complexity of tumor infiltrating CD8 T cell states is still not fully elucidated. To delineate the landscape of tumor infiltrating CD8 T cells in an unbiased manner, we collected and analyzed single-cell RNA-Seq data of CD8 T cells infiltrating mouse and human melanomas. Our unsupervised approach revealed the presence of multiple distinct CD8 T cell subtypes including exhausted, terminal effector, naive and memory-like CD8 T cells, as well as novel intermediate states, that we validate with flow cytometry. TCR reconstruction and pseudotime ordering enabled us to follow the differentiation process of single clones and suggested two distinct trajectories towards exhaustion. Altogether, this work represents the first fully unbiased characterization of CD8 T cell heterogeneity and differentiation in the tumor micro-environment, and provides an important baseline towards interpreting changes in the tumor micro-environment in response to immuno-therapy.

079

c-MET dependent neutrophil responses limit cancer immunotherapy

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The HGF/c-MET signaling pathway is dysregulated in many solid cancers. Currently inhibitors of c-MET are used in the clinic to target oncogenic signaling in tumor cells. However, the therapeutic benefit is limited to subgroups of patients with certain cancer entities. As the HGF/c-MET signaling pathway also participates in the regulation of immune responses, we hypothesized that c-MET

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inhibitors could increase the efficacy of cancer immunotherapies.
Here, we demonstrate that concomitant c-MET inhibition promoted immunotherapies in different murine cancer models independent of tumor cell´s dependence on c-MET signaling. We unraveled, that HGF/c-MET signaling in neutrophils promoted their reactive mobilization from the bone marrow and their recruitment into tumors and draining lymph nodes in response to immunotherapies. In T-cell inflamed tissues, neutrophils rapidly acquired immunosuppressive properties and thereby restrained T cell proliferation and effector functions. Concomitant c-MET inhibition impaired the reactive mobilization of neutrophils and their recruitment into tumors and lymph nodes. Finally, this promoted the expansion of anti-tumoral T cells and improved responses to cancer immunotherapy. Importantly, we also observed high serum levels of the c-MET ligand HGF and increasing neutrophil counts in the blood of melanoma patients not responding to anti-PD1 immunotherapy.
In conclusion, we identified an unknown role for the HGF/c-MET signaling pathway in the recruitment and function of neutrophils. Our data suggest c-MET inhibition as a concomitant treatment strategy to increase the efficacy of cancer immunotherapies. As reactive neutrophil responses are also seen in patients undergoing immunotherapy, this strategy could have a broad clinical applicability and may improve responses in a variety of patients.

080

The association between the local immunological microenvironment and the local microbiome in colorectal cancer liver metastases

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Introduction: Immunotherapy and cancer immunology have major impact on the clinical and therapeutic options in many cancers. Colorectal cancer has been shown to be under immunosurveillance, but immunotherapy has shown little success. On the other hand, Fusobacterium nucleatum and other beacteria have been shown to modulate the microenvironment. Therefore an in-depth analysis of the local immunological microenvironment coupled to an in-depth analysis of the tissue specific microbiome was performed.
Material and methods: Samples from metastatic colorectal cancer were obtained as described previously. Tissue was prepared and analyzed for the presence of bacterial species via specific probes (e.g. EUB338, FUS664) or cultivation or eubacterial PCR. In addition, immune cell subtype distributions and cytokines were determined. Together with follow-up and further clinical data, this dataset allowed to investigate the complex relationships between tissue microbiome and immunological composition.
Results: Analyses revealed complex patterns of associations between the presence of bacterial species and the distribution patterns of immune cells. The local immunological microenvironment is associated with and influenced by bacterial presence. Distinct patterns for specific regions (i.e. the adjacent normal liver) highlight the localization-dependent role of specific sub-tissue microenvironments. Further studies are needed to better appreciate the existing immuno-bacterial subgroups in advanced colorectal cancer.
Conclusion: Distinct patterns reveal a tight relationship between microbiome und immunological behaviour and allow further stratification and also hold the potential to improve translational efforts in modulation of the immune response against tumor cells.

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081

MDSC and neutrophil recruitment and function in melanoma primary and metastatic lesions

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Despite recent improvement in the treatment of metastasizing malignant melanoma due to the approval of PD-L1 and CTLA-4 checkpoint inhibitors, it remains a devastating disease with rapid progression, formation of metastases and poor prognosis. In addition, a continuous, stable tumor control is often hampered due to different immunosuppressive mechanisms mediated largely by myeloid-derived suppressor cells (MDSC), which fail to differentiate under chronic inflammatory conditions. These cells are recruited from the bone marrow and activated through several melanoma-derived factors, including IL-1, VEGF, IL-6 and GM-CSF and consist of monocytic (M-MDSC) and polymorphonuclear (PMN-MDSC) cells, which inhibit T and NK cell activities via various mechanisms. MDSC have been shown to accumulate in melanoma-bearing *ret* transgenic mice and melanoma patients and are associated with a higher tumor burden and worse prognosis. Since recent publications also reported this association regarding neutrophils, a high immunomodulatory capacity of both cell types is assumed.
Tumor-associated neutrophils (TAN) exist in different states of activation and differentiation, displaying either anti-tumor (N1) or tumor-promoting properties (N2). It has been shown that circulating neutrophils can be further classified based on their cellular density as anti-tumor high density neutrophils (HDN) or tumor-supportive low density neutrophils (LDN).
To improve the understanding of these heterogeneous cell populations and their contribution to tumor development, we aim to investigate the recruitment of these cells as well as their immunosuppressive function in primary compared to metastatic lesions in a *ret* transgenic melanoma mouse model mimicking the human melanoma situation as well as melanoma patient samples. Flow cytometry analysis is used to investigate the expression of immunosuppressive molecules, e.g. PD-L1, arginase-1, IDO, CD39/CD73 and production of NO and ROS by MDSC and neutrophil subsets as well as markers for their identification.
We could demonstrate an accumulation of M-MDSC and PMN-MDSC in bone marrow, spleen, lymph node and tumor tissue of tumor-bearing mice compared to wild-type controls. Both MDSC populations expressed PD-L1 and chemokine receptor CCR5, which is associated with stronger immunosuppression in MDSC, to approximately the same extent. In addition, comparative micro-array transcriptome analysis and qPCR will be performed on these myeloid cell populations. Their functional characterization will be done *ex vivo* by testing the immunosuppressive potential using T cell proliferation assays and the migration potential in chemotaxis assays.

082

***Size matters:* The novel immune checkpoint CEACAM1-3S isoform drives NK cell-mediated cytolysis and overall survival in melanoma patients**

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CEACAM1 is a widely expressed multi-functional cell-cell adhesion protein and was reported as a strong clinical predictor of poor prognosis in patients with malignant melanoma. However, the precise function of its four splice variants for disease progression and its clinical consequences is completely unknown. Although CEACAM1 has been controversially discussed as tumor suppressor but also as driver of invasion, we show that expression of CEACAM1-3S, CEACAM1-3L, CEACAM1-4S and CEACAM1-4L contrary impact melanoma progression and immune-surveillance in a variant-specific mode of action. In contrast to CEACAM1-4S and CEACAM1-4L, expression of CEACAM1-3S and CEACAM1-3L is induced during disease progression and correlates significantly with clinical stage. Surprisingly, overall survival was prolonged in patients with advanced melanomas expressing CEACAM1-3S. We provide strong evidences that the favourable role of CEACAM1-3S expression appears due to its inhibitory function on melanoma cell aggressiveness and as result of enhanced immunogenicity, modulated via up-regulation of cell surface expressed ligands for the NKG2D receptor, sensitizing these cells to natural killer cell-mediated cytolysis. Contrarily, expression of CEACAM1-4L down-regulated cell surface levels of MICA and ULBP2 by enhanced shedding, thus, supporting a tumor progressive phenotype. These results highlight the significance of the variant-specific immunomodulatory and cell biological functions of CEACAM1 in melanoma pathogenesis. Furthermore, we identified MITF, the master regulator of melanocyte differentiation and melanoma oncogene, as a direct regulator of CEACAM1 expression in melanoma cell lines and tissue. Moreover, Cancer Genome Atlas (TCGA) database-based analyses revealed significant correlation of MITF and CEACAM1 expression in patient-derived melanoma tissues. Taken together, these novel mechanistic insights into CEACAM1 function as well as the regulation of its expression might help to decipher new targets for the development of innovative therapeutic strategies for the treatment of malignant melanoma.

083

Oncogene-specific T cells fail to eradicate lymphoma-initiating B cells

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To date, little is known about the interaction between (pre-)malignant B cells and T cells. We generated transgenic mice that allow B cell-specific induction of the oncogene SV40 large T-antigen (TAg) to analyze the role of oncogene-specific T cells during sporadic B cell lymphoma development. Constitutive TAg expression in CD19-Cre x LoxP-Tag mice resulted in TAg-tolerant $CD8^+$ T cells and development of B cell lymphomas. In contrast, CD19-$CreER^{T2}$ x LoxP-Tag mice retained TAg-competent $CD8^+$ T cells at time of oncogene induction and TAg expression in few B cells of adult mice resulted in exceptionally rare lymphoma formation late in life. Increased lymphoma incidence in the absence of TAg-specific T cells suggested T cell-mediated inhibition of lymphoma progression. However, TAg-initiated B cells were not eliminated by T cells and detected long-term. Our results demonstrate a failure of the immune system to eradicate lymphoma-initiating B cells, retaining the risk of lymphoma development.

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Role of tumor-derived extracellular vesicles in immunosuppression in malignant melanoma patients

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Malignant melanoma is one of the most dangerous forms of skin cancer and accounts for majority of all skin cancer deaths. The accumulation of highly immunosuppressive regulatory leucocytes, especially myeloid-derived suppressor cells (MDSC), plays a significant role in resistance to immunotherapy of malignant melanoma. Extracellular vesicles (EVs) are membrane-bound carriers with complex cargos containing proteins, lipids, and nucleic acids. They include microvesicles, exosomes and apoptotic bodies. Tumor-derived EVs can promote the progression, invasion and metastasis of cancer. In particular, they can trigger cytokines and chemokine production by immune cells. However, the role of tumor-derived EVs in immune suppressive mechanisms in malignant melanoma and its interaction with MDSCs remain to be explored. The aim of this investigation is to study molecular mechanisms of interactions of tumor-derived EVs with myeloid cells in melanoma patients leading to their conversion into MDSC and to further stimulation of their immunosuppressive functions. We found that tumor-derived EVs can induce the anti-apoptosis ability of human $CD14^+$ monocytes from healthy donor via the upregulation of BCL-2. Moreover, they could upregulate the PD-L1 expression and activate the NF-κB signaling pathway in these cells, which is mediated by toll-like receptor (TLR) 4. In addition, specific miRNA were shown to be inserted into tumor-derived EVs and taken up by immature myeloid cells. We found also that melanoma-derived EVs expressed proteins and miRNA with regulatory functions. We suggest that EV measurement will help to establish their prognostic value in melanoma patients treated with various immunotherapies.

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Targeting CD70-positive cancer associated fibroblasts to tackle the immune suppressive tumor microenvironment in colorectal cancer

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Introduction: Tumor progression and invasiveness are determined not only by the malignant cancer cells themselves but also by the surrounding tumor microenvironment, comprising of cancer-associated fibroblasts (CAFs). CAFs represent a heterogeneous population with both cancer-promoting and cancer-restraining actions, lacking specific markers to target them. Expression of the immune checkpoint CD70 is normally tightly regulated and limited to cells of the lymphoid lineage. Instead, tumors hijack CD70 to facilitate immune evasion by increasing the amount of suppressive regulatory T cells (Tregs), inducing T cell apoptosis and skewing T cells towards T cell exhaustion. In this study, we aimed at exploring the expression patterns of CD70 in colorectal cancer (CRC), not merely focusing on the tumor cells, but also taking the tumor microenvironment into account.
Material and methods: We have analyzed the prognostic value of CD70 expression by immunohistochemistry in CRC specimens and studied its relationship with well-known fibroblast

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markers, microsatellite instability and Tregs. Furthermore, primary CAF cell lines were used to study the role of CD70 on migration and immune escape.
Results and discussion: We revealed prominent expression of CD70 on a specific subset of CAFs in invasive CRC specimens. Cancer cells show almost no expression of CD70. $CD70^+$ CAFs proved to be an independent adverse prognostic marker. Functionally, $CD70^+$ CAFs stimulated migration and significantly increased the frequency of naturally occurring Tregs and production of interleukin-2. Finally, experiments aimed at therapeutically targeting these CD70-positive CAFs are currently being analyzed using 2D and 3D models.
Conclusion: We have identified the expression of CD70 on CAFs as a novel prognostic marker for CRC. Performing this research, we found evidence of a cross talk between $CD70^+$ CAFs and naturally occurring Tregs, paving the way towards immune escape. As such, this study provides a strong rationale for our ongoing exploration of CD70-targeting antibodies in CRC, especially in light of the limited immunotherapeutic options available in CRC.

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The macrophage immune checkpoint CD47 is a direct target of EMT transcription factors SNAI1 and ZEB1 and its blockade activates the phagocytosis of breast cancer cells undergoing EMT
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The macrophage immune checkpoint CD47 is a cell surface transmembrane protein expressed on the surface of tumor cells. By binding to its ligands, signal regulatory protein α (SIRPα) and thrombospondin-1 (TSP-1), on the surface of macrophages and dendritic cells, CD47 delivers a strong “don't eat me signal” to block the phagocytosis of cancer cells. Therefore, blockade of CD47 by using anti-CD47 monoclonal antibodies increased macrophage- and T cell-mediated phagocytosis and elimination of various cancer cells. In this study, we investigated the mechanism underlying the expression of CD47 in highly aggressive breast cancer cells undergoing Epithelial-to-Mesenchymal Transition (EMT). We showed that CD47 was upregulated in different EMT-activated human breast cancer cells versus epithelial MCF7 cells. Overexpression of EMT transcription factor SNAI1 or ZEB1 in epithelial MCF7 cells activated EMT and upregulated CD47 expression while siRNA-mediated targeting of SNAI1 or ZEB1 in mesenchymal MDA-MB-231 cells reversed EMT and strongly decreased CD47 expression. Mechanistically, SNAI1 and ZEB1 upregulated CD47 by binding directly to E-boxes in the human CD47 promoter. TCGA and METABRIC data sets from breast cancer patients revealed that CD47 correlated with SNAI1 and the EMT marker Vimentin. At functional level, different EMT activated MCF-7 breast cancer cells were less efficiently phagocytosed by macrophages compared to parental epithelial MCF7 cells. The phagocytosis of EMT-activated cells was rescued by using CD47 blocking antibody or by genetic targeting of SNAI1, ZEB1 or CD47. These results provide a rationale for the use or combine anti-CD47 blocking antibody in patients with highly aggressive, mesenchymal, and metastatic breast cancer.

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Therapy resistance in renal cell carcinoma correlates with an endothelial cell dependent altered immune cell infiltrate
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Renal cell carcinoma (RCC) is characterized by a suppressive immune cell infiltrate. Our objective was to investigate leukocyte adhesion to vascular endothelium after conditioning with untreated and therapy-resistant RCC cells. Untreated (parental) and everolimus-resistant 786-O and KTCTL-26 cells were co-cultivated with microvascular endothelial cells (HMEC). HMEC without tumor cell contact served as controls. Subsequently, leukocyte adhesion to HMEC was evaluated. Subtypes of bound leukocytes and the endothelial adhesion receptors, involved in the attachment of specific subtypes, were analyzed. Co-cultivation of HMEC with parental 786-O and KTCTL-26 resulted in an altered CD4+/CD8+ immune cell ratio adhering to HMEC, preferring attachment of CD4+ cells (KTCTL-26 > 786-O). Concurrently, the count of CD56+ natural killer (NK) cell binding to tumor cell conditioned HMEC decreased. Incubation with therapy-resistant RCC cells contributed to a switch of the CD4+/CD8+ immune cells ratio towards CD8+ cells attaching to HMEC, whereas the amount of CD56+ NK cells was further reduced. Moreover, the percentage of activated NK cells (CD45R0+) adhering to HMEC was significantly reduced after pre-conditioning with therapy-resistant RCC cells compared to parental cells. At the same time, CD4+ cells bound in particular to endothelial E-selectin, weaker to ICAM-1 and VCAM-1. CD8+ bound the strongest to ICAM-1, CD16+ NK cells mainly to VCAM-1 and E-selectin and CD56+ NK cells only to ICAM-1. Due to our data we conclude that the occurrence of therapy resistance in RCC cells causes conditioning of the vascular endothelium, leading to enhanced adhesion of CD8+ immune cells and impaired binding of activated NK cells.

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B cells and the antibody response in melanoma: mechanisms of immune modulation and translational opportunities

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B cells have been reported to contribute different and often controversial roles in cancer, ranging from tumour immune escape to production of protective anti-tumour antibodies. We examined the humoral immune compartment in malignant melanoma. Mature B cells in patient circulation could express IgG antibodies that recognized melanoma cells. When studied *ex vivo,* these antibodies could mediate tumour cell cytotoxicity. We detected a tissue-resident mature B cell compartment displaying classic hallmarks of clonal expansion, class switching and antibody affinity maturation in melanoma. On the other hand, tumour-associated B cells expressed proportionally lower immune activatory antibody isotype IgG1 levels when compared to B cells from lymph nodes and blood. Importantly, tumour-associated Th2-biased inflammatory conditions, featuring enhanced expression of IL-10, IL-4 and VEGF, could influence tumour-associated B cell isotype switching in favour of immunologically-inert IgG4 antibodies. A tumour antigen-specific IgG4 antibody significantly reduced the potency of a normally cytotoxic anti-tumour IgG1 *in vivo*, suggesting immune impairing functions for IgG4. In the blood of patients with melanoma, proportionally higher IgG4 levels were associated with disease progression and worse patient prognosis. Our findings suggest that B cells and their expressed antibodies may participate in inflammation and immune escape in melanoma and could influence the cancer immune landscape. Translational opportunities from our findings may be derived by identification of humoral immune markers as prognostic or monitoring tools, as well as through the design of therapeutic agents including monoclonal antibodies less prone to tumour-associated immunosuppressive forces.

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In vivo siRNA knockdown of Wnt1 rescues dendritic cells from b-catenin activation and sensitizes tumors to vaccination

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Background: Aberrant WNT-b-catenin signalling is associated with several types of cancers by increasing cancer cell proliferative, metastatic potential and stemness. Recent melanoma studies further suggest a profound immunosuppressive effect of intratumoral WNT-b-catenin pathway in dendritic cells (DCs). There are two important caveats in exploiting the WNT pathway: it plays a crucial role in homeostasis and there are 19 human WNT ligands which are found differentially expressed in several human cancers. Targeting upstream the WNT signalling cascade the specific cancer cell-derived ligands that induce WNT pathway-dependent immunosuppression is expected to be a safer and more effective immunotherapeutic approach than targeting its intracellular counterparts.
Methods: We have set-up a combinatorial experimental approach that integrates primary human tumor analysis and syngeneic tumor models to identify cancer cell-derived WNTs that could become targets for a next-generation immunotherapeutic trials.
Results: Firstly, we analysed the human Cancer Genome Atlas (TCGA) database and an in-house biobank of human lung adenocarcinoma to investigate correlations between WNT1-19 ligand expression and expression of immunosuppressive genes (RNA level) or numbers of T cytotoxic cells (IHC). WNT1 showed the strongest correlations. Silencing human WNT1 in dissociated human lung adenocarcinoma cultures confirmed its immunosuppressive properties. We therefore created a Wnt1-overexpressing and a WNT1-silenced murine cancer cell line, which we used in two syngeneic tumor transplantation models (subcutaneous/intrathoracic). Murine lung adenocarcinoma in immunocompetent, but not in immunodeficient mice, was strongly dependent on WNT-1. We observed strong tolerogenic effects of WNT1 on cancer antigen-specific T cells, such as low proliferation, activation, in vivo cytotoxicity and impaired immunological memory. Using Wnt pathway-reporter mice and an inducible DC-knock-out mouse model, we further show that WNT1-exposed DCs are sufficient and required for failure of adoptive T cell therapy. Pre-clinical trials showed that WNT1 siRNA-loaded nanoparticles rescued intratumoral DCs from b-catenin activation and acted in synergy with DC-targeted vaccination.
Conclusions: Our studies unravel the crucial role of WNT1 in adaptive immune resistance of solid tumors and raise hopes that if in vivo RNA interference against WNT1 is safe in humans it could give excellent therapeutic responses.

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Indoleamine 2,3-dioxygenase 1 (IDO1) in human T cells is inducible upon T cell activation and type I interferons

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Background: The protein indoleamine 2,3-dioxygenase 1 (IDO1) is known to accomplish immune regulatory effects in physiological as well as pathophysiological processes. IDO1 is the rate-limiting enzyme within the tryptophan catabolism, which results in secretion of immunosuppressive kynurenines. A second mechanism involves its ITIM motives that recruit and activate downstream tyrosine phosphatases (SHP-1/SHP-2), leading to the activation of the NF-kB pathway. Both, enzymatic as well as signaling properties induce a regulatory immune profile and have been linked to

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an immunosuppressive tumor milieu.
Methods: Human T cells were MACS-isolated from peripheral blood mononuclear cells and either left untreated or stimulated with aCD3/aCD28 T cell activation beads, or interferon. IDO1 mRNA expression was assessed by qRT-PCR and protein expression was acquired by Western Blot analysis using the human-specific D5J4E clone.
Results: We found that IDO1 is inducible in primary human T cells upon T cell activation as well as upon type I and type II interferon signaling. Interestingly, minimal IDO1 expression was already detectable after T cell isolation, which decreased within 24 h of T cell culture. Besides IFN, IDO1 induction could be provoked by various other stimuli including different interleukins. Of note at this point, cytokine-induced IDO1 induction did not alter proliferative capacity of bead-stimulated T cells.
Discussion: Our data show for the first time that IDO1, an ancient well-described enzyme in antigen-expressing cells, is expressed in human T cells. Having the early and - so far -unspecific induction of IDO1 after cell isolation in mind, one might speculate possible implications in an exhaustive T cell phenotype, following adoptive cell therapy (ACT). Therefore, tackling IDO1 functions in T cells might be a so far unrecognized strategy in the immunotherapy of cancer.

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RIG-I-like helicases signaling induced alteration of myeloid-derived suppressor cells phenotype and function unleashes tumor immune control in murine pancreatic cancer

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Background: RIG-I-like helicases **(**RLH) activate a type I IFN-driven immune response and induce an immunogenic form of tumor cell death in pancreatic cancer, thereby bypassing tumor-mediated immunosuppressive mechanisms. Amongst others the dense infiltration with cells of the myeloid lineage, such as macrophages and myeloid-derived suppressor cells (MDSCs), entertains an immunosuppressive machinery in the tumor microenvironment. This has been linked with a dismal prognosis of patients with advanced pancreatic cancer. Undifferentiated, immature cells of monocytic (M-MDSCs) and polymorph nuclear (PMN-MDSCs) origin in the tumor impair T cell function and promote tumor growth. Overcoming immune escape is the focus of various strategies in tumor therapy. Here we assess the effect of treatment with the synthetic melanoma differentiation antigen 5 (MDA5) ligand polyinosinic:polycytidylic acid (poly(I:C)) on MDSC phenotype and function in tumor and spleen.
Methods: C57BL/6 and IFNAR$^{-/-}$ mice were orthotopically injected with T110299 pancreatic cancer cells, derived from a GEMM tumor (KPC), treated i.v. with poly(I:C) complexed with invivo jet-PEI and sacrificed after 21 days. PMN-MDSCs (CD11b^{+} Ly6G^{+} Ly6C^{int}) and M-MDSCs (CD11b^{+} Ly6G^{-} Ly6C^{+}) from tumors and spleen were isolated for flow cytometry, RNA-Sequencing and functional assays. RNASeq data were subjected to KEGG Pathway, CIBERSORT and Gene Set Enrichment Analysis. Key findings were validated in KPC mice.
Results: Survival of poly(I:C)-PEI treated mice with orthotopic pancreatic tumors was significantly prolonged. After only two injections of poly(I:C)-PEI a reduction in tumor size was observed. RNA-Sequencing showed a predominant change of IFN regulated genes to be altered in MDSCs. Midst other pathways KEGG-Analysis revealed certain members of antigen presentation and processing to be modulated on transcriptional level. MHC-I-pathway was induced, whereas MHC-II-pathway was decreased. Flowcytometry backs up the upregulation of MHC-I and CD86 and reduced MHC-II in MDSC-subpopulations. In IFNAR$^{-/-}$ mice expression levels were unaltered pointing towards a type I IFN-mediated effect. MDSCs of poly(I:C)-treated mice lost their suppressive function on antigen independent CD8^{+} T cell proliferation *in vitro*. RNA-Sequencing data are currently being evaluated in regards to potential suppressive mechanisms. CIBERSORT-Analysis pointed out a reduction of M2 phenotype paralleled by increased expression levels of M1 genes in bulk tumor tissue after therapy.
Conclusion: Survival benefit of RLH ligand treated mice with pancreatic cancer correlated with reduction in tumor size, reduced T cell suppressive capacity of MDSC and functional reprograming

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towards a M1/G1 phenotype. Type I IFN signaling induced by RLH-based immunotherapy has the potential to revert tumor induced immune suppression for unleashing the potential of tumor-resident CTL for effective immune control.

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Cytomegalovirus infection leads to c-MET overexpression, adversely affecting survival and resistance to Temozolomide in glioblastoma

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Objective: Cytomegalovirus (CMV) is detectable in most, if not all human glioblastoma samples. The receptor tyrosine kinases c-MET and its co-receptor CD44 are associated with shorter survival time and poor treatment response to the standard of care drug temozolomide (TMZ) in glioblastoma. While CMV can affect various oncomodulatory signalling pathways, its role in glioblastoma remains largely elusive. Here, we investigate the interrelation of CMV interference with the nuclear factor (NF)-kB pathway in the regulation c-MET/ CD44 complex and mediation of TMZ resistance.
Methods: Changes in c-MET and CD44 expression after CMV infection were measured using qPCR and Western blot analysis. The involvement of the NFkB pathway was determined using siRNA in GSCs. Functional analysis was performed using different mouse models *in vivo.*
Results: Detection of the CMV major immediate-early gene 1 in patient-derived tumour samples correlates linear with c-MET receptor expression (n=15, p=0.0005, R^2=0.748). CMV infection of patient derived glioma stem cells (GSCs) leads to upregulation of c-MET/ CD44 complex on mRNA and protein level. This upregulation is more pronounced in low c-MET/ CD44- expressing, proneural GSCs (95-fold -/+ 9.3), compared to those of the mesenchymal subtype (2-fold-/+ 0.16). Additionally, an upregulation of the c-MET receptor phosphorylation is detected in western blot analysis, suggesting an activation of the c-MET signalling pathway in these cells. The knock-down of the NF-kB p65 subunit using siRNA prevents c-MET upregulation. Phenotypically, a stable overexpression of c-MET on proneural GSCs leads to increased migration *in vitro* and shortened survival time in an orthotopic mouse xenograft model *in vivo* (mean survival: 10d vs 60d, p < .0001).
Latent CMV infection in a syngenic mouse glioblastoma model leads to c-MET overexpression in tumour cells, shortened survival (34d vs 40d, p=0.0004) and increased TMZ resistance (mean survival: 35d vs 48d, p= 0.0023). Treatment with the antiviral drug ganciclovir partially reverse CMV induced TMZ resistance (40d vs 49d, p < 0.0001).
Conclusion: CMV increases glioblastoma progression by upregulation of growth and invasion mediating receptor c-MET/CD44 complex. *In vivo*, shortened survival times and increased resistance to TMZ can be antagonised by antiviral therapy. These findings further strengthen the importance of CMV infection glioblastoma and suggest a potential role for supportive antiviral therapy.

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Investigating the feasibility of tumour infiltrating lymphocyte therapy for paediatric malignancies with high risk and poor prognosis

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Background: Brain tumours are the most common solid malignancy of childhood, accounting for >20% of all paediatric cancers. Collectively, they remain the leading cause of cancer-related death and long-term morbidity in children.
With current practice, adjuvant therapy has helped to improve cure rates for certain brain tumour subtypes, such as medulloblastoma. However, using conventional chemotherapy and irradiation to achieve such success is typically achieved with a significant burden to the survivor. Improvements in survival for many other childhood brain tumours have not been observed despite a spectrum of multimodal therapies.
Tumour infiltrating lymphocyte (TIL) therapy consists of extracting immune cells from surgically removed tumours and growing them in the lab. This not only allows immune cells to be “switched back on”, but increases their total number. In the setting of metastatic melanoma, re-infusing these cells into patients has led to long term tumour remissions. In this study we are investigating whether applying TIL therapy to paediatric brain tumours is feasible.
Objectives: We seek to assess:
1. If there are significant T-cell infiltrates in high grade paediatric brain tumours
2. Whether these cells can be efficiently expanded *ex vivo*
3. The anti-tumour reactivity of expanded TILs against autologous tumour *ex vivo*
Results: We report initial evidence that there is a significant presence of TILs in 4 high grade paediatric brain tumour patients with cell type and phenotype analysed by time of flight cytometry (cyTOF) upon dissociation after resection and after 3 weeks expansion in IL-2
Initial samples have displayed up to 1300 fold expansion of TILs upon 3 weeks of culture and cyTOF analysis has shown that expanded cells have an increased capacity to secrete effector cytokines compared to peripheral blood lymphocytes cultured in the same conditions. Crucially, multiplex analysis of supernatant following co-culture of with autologous tumour and tumour lines shows that expanded cells possess anti-cancer activity.
Conclusions: These promising results suggest that TIL therapy for paediatric brain tumours may be feasible. Analysis of an increased number of samples will be required to substantiate this, along with optimisation of methodology to produce clinically relevant numbers of cells.

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Omental fat in ovarian cancer induces lymphangiogenesis

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Ovarian cancer metastasis occurs by direct multifocal seeding into the peritoneum as well as by migration through the lymphatic system. High grade ovarian carcinoma patients present with distant metastases. Significant risk factors for the development of those are stage, grade, and lymph node involvement. The increase of the number of lymphatic vessels seems to be implicated in ovarian tumor progression. While the tropism of ovarian cancer cells for fat is well described, the potential impact of an adipose-rich microenvironment on the dissemination of metastasis via lymphatic vessels has never been investigated.
In this study, we examined the effect of omental fat on lymphangiogenesis in ovarian carcinoma. For that we used a cohort of 50 ovarian cancer specimens. We observed a higher number of tumor-associated vessels and principally lymphatic vessels in ovarian cancer in contact with the omentum. These lymphatic vessels are predominantly localized along the fat tissue. A higher secretion of VEGF-C is observed in ovarian tissues containing fat compared to the ones without fat giving a potential explanation to the observed increase of lymphatic vessels in fatty tissues. We also developed a healthy fat tissue explant culture model and treated whole tissue explants with ascites. Herein, we

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observed an increase of the number of adipose-derived stem cells (ADSCs). These ADSCs express lymphatic markers such as D2-40 and Lyve-1. Comparatively, when we isolate ADSCs we observed an expression of lymphatic markers after treatment with ascites.
In conclusion we can say that omental fat in ovarian cancer seems to have an impact on lymphangiogenesis. The close contact of ascites with fat tissue seems to lead to a differentiation of adipose-derived stem cells into lymphatic endothelial cells. Further investigations must be performed to understand the exact mechanisms underlying this phenomenon.

The first two authors on this abstract have an equal contribution in this work.

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Pancreatic tumor-derived factors induce expansion of myeloid cells with high suppressive capacity

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Background: In the last decade, cancer immunotherapy exhibited promising improvements; however, pancreatic cancer remains a major challenge. Myeloid cells such as myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) are major contributors to the immunosuppressive tumor milieu. Tumor-derived factors are known to manipulate the immune system locally as well as systemically to escape immune surveillance. In this study, we aim at identifying tumor-driven mechanisms, which regulate myeloid cell differentiation and contribute to the immunosuppressive microenvironment.
Methods: Standard GM-CSF bone marrow cultures were supplemented with KPC (Kras/p53/Cre) pancreatic tumor-conditioned medium to study myeloid cell differentiation. Steady state levels of tumor-secreted factors, such as GM-CSF and G-CSF, were measured by ELISA. Suppressive capacity was analyzed as inhibition of T cell proliferation. To assess myeloid cell differentiation *in vivo*, KPC-derived tumor cells were implanted orthotopically into the pancreas and immune cell compositions in blood, spleen and tumor were analyzed by flow cytometry. RNA from FACSsorted MDSC of tumor-bearing and tumor-free mice was isolated and sequenced using next generation sequencing (RNAseq). Differential expression analysis will be performed to indentify tumor-induced transcriptional programs.
Results: Tumor-conditioned medium strongly inhibited dendritic cell (DC) differentiation, favoring the enrichment of $CD11b^+$ $Gr1^+$ MDSC-like cells. Of note, steady state G-CSF levels in tumor supernatants significantly correlated with the frequency of PMN-MDSC. We identified the $CD11b^+$ $Gr1^+$ MHC-II^{neg} cell population as the main driver of T cell suppression. *In vivo,* KPC-derived tumors induced the expansion of the myeloid cell compartment in both blood and spleen. All tumors investigated were highly infiltrated with heterogeneous myeloid cell populations with PMN-MDSC ($CD11b^+$ $Ly6G^+$ $Ly6C^{int}$) and TAM ($CD11b^+$ $Ly6C^{low}$ $F4/80^+$) as major constituents. Interestingly, G-CSF serum levels of tumor bearing mice were elevated; however GM-CSF was not detectable.
Discussion: Here, established a robust model to study MDSC differentiation and function *in vitro*. We showed that tumor-secreted factors strongly inhibited DC differentiation and drive MDSC expansion. Furthermore, tumor-derived factors induced a suppressive program in myeloid cells that efficiently inhibited T cell proliferation. Preliminary data show that G-CSF, but not GM-CSF, might be the major driver of PMN-MDSC expansion in pancreatic tumor-bearing hosts.

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Investigation of ICOS expression on human and mouse T cell subsets
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The existence of constant immuno-editing following therapy administration requires the investigation of novel immuno-regulatory targets to prevent immune escape. Besides blocking inhibitory immune pathways, the activation of effector immune cells emerges as a promising anti-cancer strategy. Along these lines, T cell responses can be amplified by providing co-stimulatory signals. One such co-stimulatory receptor is the inducible T cell co-stimulator (ICOS), which belongs to the CD28 family and is described to be up-regulated on T cells upon activation through TCR engagement and further upon binding to its ligand B7H2. However, the direct comparison of ICOS expression to other immunomodulatory T cell receptors on healthy as well as on tumor-derived material has not been investigated yet and might provide important insight for cancer therapy development. To address this, we studied the kinetics of ICOS expression upon various primary stimuli on different T cell subsets and assessed how it compares to the expression of members of the tumor necrosis factor receptor superfamily including 4-1BB Ox40 as well as to members of the immunoglobulin-like superfamily like PD-1, or CTLA-4.
We observed that ICOS is expressed at low levels on resting naive T cells (highest on Tregs > CD4+ > CD8+ T cells) and is rapidly up-regulated to similar levels on all T cell subsets following T cell activation. Notably, the expression and up-regulation on mouse and human T cells is comparable. ICOS up-regulation is faster and long-lasting (even after removing the primary stimulus) compared to other T cell co-stimulatory receptors. In a next step we analyzed ICOS expression on tumor infiltrating T cells (TILs) derived from patient derived tumor samples and found higher levels of ICOS expression compared to T cells derived from normal adjacent tissue or peripheral blood of healthy donors. In addition, ICOS expression is higher on human TILs compared to 4-1BB, CTLA-4 or Ox40.
We found the same trend on mouse TILs, where ICOS expression is increased compared to T cells from secondary lymphoid organs. This is accompanied by an increased co-expression of PD-1, Ox40 and CTLA-4 correlating with increased tumor burden. In the presence of a simultaneous anti-CD3 trigger, co-stimulation of ICOS induces T cell proliferation, as well as T cell differentiation towards central and effector memory subsets. The increase in number of T cells but also the development of T cell memory upon ICOS co-stimulation may help to drive long-lasting anti-tumor effects.
In summary, ICOS expression appears to have an important role in T cell co-stimulation due its fast up-regulation and long lasting expression. Its high expression on TILs compared to other receptors together with the fact that ICOS agonism induces T cell proliferation and differentiation makes ICOS a highly interesting target for cancer immunotherapy.

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Acute myeloid leukemia (AML) promotes generation of iTregs
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Acute Myeloid Leukemia (AML) is the most common form of acute leukemia in adults. AML patients exhibit an immunosuppressive environment. Foxp3$^+$ T regulatory cells (Tregs) have been implicated as a contributor to the immunosuppressive conditions observed in AML. Foxp3 is a transcription factor that is required for the differentiation, function, and maintenance of Tregs. Under normal conditions, Foxp3$^+$ Tregs play a pivotal role in the maintenance of immunological self-tolerance and homeostasis. However, cancer cells are known to use tactics to escape immune control, one of which is the upregulation of Tregs to suppress anti-tumor effector cells. It is known that the frequency of Foxp3$^+$

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Tregs is higher in AML patients, and this is associated with poor prognosis.
In this study, we discovered that an M5 (monocytic) AML cell line, THP-1, effectively induced Foxp3$^+$ Treg differentiation *in vitro*. Expression of Foxp3 by T cells stimulated in the presence of THP-1 is maintained over 2 weeks. Moreover, these T cells showed potent immune suppressive functions *in vitro*. These data suggest that THP-1 induce functional Foxp3$^+$ Tregs by direct interactions with naïve peripheral T cells.
Neuropilin-1 (NRP-1) has recently been described as a potential therapeutic target for multiple types of cancer, including AML, as it has been observed to be elevated in the tumor environment. Our data show a positive correlation between NRP-1 expression level by THP-1 and the efficacy of Treg induction. NRP-1 can bind active and latent transforming growth factor-β (TGF-β), which is an essential factor for Treg induction. Together, these data implicate that AML creates an immunosuppressive environment by inducing the differentiation of Foxp3$^+$ Tregs via a NRP-1 dependent mechanism. Currently, we are testing the role of NRP-1 in THP-1-induced Foxp3$^+$ Treg differentiation to understand how AML promotes immune suppression in patients.

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Urokinase type Plasminogen Activator and Beta-galactoside alpha 2,6 sialyltransferase 1 found in cancer cell exosomes cause autocrine release of CXCR2 ligands

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Tumor-derived factors are often involved in resistance to conventional and targeted treatments. We previously found that factors released by metastatic breast carcinoma cells increased MIP2 (Macrophage Inflammatory Protein 2), a chemokine ligand for CXC receptor 2 (CXCR2). CXCR2 antagonists are in clinical trials for various diseases including cancer. Hence tumor derived factors by increasing endogenous ligand of CXCR2 may lead resistance to CXCR2 antagonists. Hence we here examined the effects of autocrine factors on MIP2 and KC (Keratinocyte Chemoattractant); the ligands for CXCR2. We previously examined exosomal contents of metastatic breast carcinoma cells as well as non-metastatic 67NR cells and found that more than eighty proteins significantly altered, mostly increased, in metastatic cells compared with non-metastatic cells. In this study we selected three of these autocrine factors to investigate their role on MIP2 and KC release. These factors were Urokinase type Plasminogen Activator (uPA), Beta-galactoside alpha 2,6 sialyltransferase 1 (ST6GAL1) and Complement component 3a (C3a).Breast cancer metastasizes mostly to liver and brain. We previously isolated liver, brain and heart metastatic cells of 4T1 murine breast carcinoma and named them as 4TLM, 4TBM and 4THM, respectively. 67NR cells were originally obtained from spontaneously formed breast tumor from which 4T1 metastatic breast carcinoma cells were obtained. Protein levels in conditioned mediums of metastatic and non-metastatic cells were verified by ELISA (for C3a and uPA) or immunoblot (for ST6GAL1). Brain (4TBM) and heart (4THM) metastatic subset of murine 4T1 breast carcinoma cells were treated with specific antagonists of uPA, ST6GAL1 and C3a (IPR-803, 3-Fax Neu 5-AC and SB290157, respectively), then changes in cell proliferation and chemokine release were determined. The effects of antagonists were evaluated in two different experiment conditions: 1. Treatments were applied in the presence of high (%5) FBS which allows fast cell proliferation, 2. Treatments were prepared in very low (%0.2) FBS which allows growth under the influence of autocrine factors. IPR-803, an antagonist of uPA, suppressed cell proliferation at 10 mMol concentration. Similarly, IPR-803 also significantly suppressed both MIP2 and KC secretion. ST6GAL1 inhibitor 3-Fax Neu 5-AC significantly suppressed cell proliferation in the presence of high FBS. 3-Fax Neu 5-AC also suppressed secretion of both chemokines. C3a levels were significantly higher in 4THM cells compared with other metastatic and non-metastatic cells. SB290157 did not altere chemokine secretion as well as cell proliferation. These results demonstrated that uPA and ST6GAL1 but not C3a may mediate chemoresistance in metastatic breast carcinoma.

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Investigation of effects of MSCs on immunoevasive adaptations of breast tumor cell lines, MDA-MB-231 and MCF-7

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Human mesenchymal stem cells (**MSCs**) are adult cells with the ability to differentiate into multiple mesenchymal lineages such as chondrocytes, osteoblasts, or adipocytes. MSCs also have strong immunomodulatory properties, such as inhibition of cytotoxic abilities of natural killer cells and CD8+ T cells. In addition to these, MSCs suppress the proliferation of peripheral blood mononuclear cells in response to specific or nonspecific stimulations and increase the $CD4^+$, $CD25^+$, $FoxP3^+$ Treg cells. Because of these strong immunomodulation properties, MSCs are used in the treatment of fatal diseases such as **GVHD** and some **autoimmune diseases**. In the last years, MSCs have been shown to be a component of the tumor microenvironment. However, presented findings of effects of MSCs on tumor immunology are controversial. In this perspective, we aimed to investigate the potential contributions of MSCs on the immune evasive adaptations **PD-L1, HLA-G and IDO-1** expressions of breast tumor cell lines MDA-MB-231 and MCF-7. In most of cell types PD-L1, HLA-G and IDO-1 expressions are dependent to IFN-g stimulus. For this purpose, we co-cultured these cells with different IFN-g concentrations and analyzed changes of PD-L1, HLA-G and IDO-1 expressions by using FACS and ELISA methods. Our preliminary results showed us, with the MSCs, breast tumor cells increased expressions of PD-L1, HLA-G and IDO-1 compared with absence of MSCs. According to our early findings, MSCs may provide an eligible environment to tumor cells for evade from immune cells.

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The effects of mesenchymal stem cell on the macrophage phenotype

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Mesenchymal stem cells (MSCs) are potent immunomodulator cells, which that clinically used in many immune system related disorders, such as GVHD, Crohn's Disease or transplantation of various graft. However, the clinical effects of MSCs generally short termed. To prolong these effect, MSCs may need to interact with macrophage cells, which orchestrator of immune response by the antigen presentation. In this study, we aimed to investigate the effects of MSCs on the phenotype changes of macrophage cells. For this purpose, peripheral blood monocytes were isolated and co-cultured with MSCs by separately stimulation of M-CSF and GM-CSF. After the stimulation and co-culture, M1 and M2 macrophage markers CD14, CD64, CD80, CD163, CD200R expressions were analyzed by flow cytometry method. According to our first findings, CD14 and CD200R expressions of stimulated macrophages were not affected with MSCs, however CD64, CD80 and CD163 expressions increased compared with unstimulated macrophage cells. These findings suggested that, the macrophages may be provoked by MSCs to differentiate into the both of M1 and M2c like phenotype. Tumor associated

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macrophages (TAMs) are a unique macrophage subset, and their phenotypes accommodates both M1 and M2 like macrophage characteristics together. Similar to our findings, as a component of tumor microenvironment, MSCs maybe contribute to developing and sustaining the TAM phenotype in the tumor tissue. In this perspective, MSCs may considered another immune evasive factor for tumor and immune cell interactions. However, our early results need to support by using large sample number, more specific markers and functionality experiments.

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Expression of MHC class I determines the prognostic benefit of tumor-infiltrating T cells in ovarian cancer independent of the PD-1/PD-L1 axis

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Purpose: The presence of tumor-infiltrating lymphocytes (TILs) is generally associated with an improved prognosis. However, we recently observed that this prognostic benefit of TILs was absent in ovarian cancer (OC) patients treated with neoadjuvant chemotherapy. Here, we therefore assessed whether tumors treated with neoadjuvant chemotherapy were characterized by loss of Major Histocompatibility Complex class I (MHC1) expression and/or upregulation of PD1/PD-L1.
Experimental design: Expression of MHC1, PD-1/PD-L1 and relevant associated immune cell markers were assessed in chemotherapy-naïve (CN) and -experienced (CE) tumors using a tissue microarray of 171 OC patients.
Results: 25.0% of CN and 1.6% of CE tumors expressed cancer-cell associated MHC1. Expression of MHC1 on stromal cells was evident in 92.1% of CN and 80.5% of CE. In stroma, expression of MHC1 was observed on both lymphocytes and myeloid cells. Expression of PD-L1 on cancer cells was focal and present in 17.3% of CN and 16.0% CE patients. As for MHC1, expression of PD-L1 in stroma reflected immune infiltrates and was observed in 23.2% of CN and 24.0% of CE patients. PD-1+ TIL were abundant in cancer islets, largely absent from stroma, and did not differ in number between CN and CE tumors. Finally, CD8+ and PD-1+ TIL were of prognostic benefit only in patients with MHC1+ tumors, independent of PD-L1 expression.
Conclusions: OC tumors exposed to neo-adjuvant chemotherapy are characterized by loss of MHC1 expression with concomitant absence of prognostic benefit of TILs. Combination strategies that induce MHC1 may be needed to facilitate chemo-immunotherapy for these patients.

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Uncovering miRNAs that affect melanoma cell recognition and killing by antigen specific CD8$^+$ T cells

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The regulatory effect of microRNAs (miRNAs) in cellular anti-tumor immune responses has been described with predominant focus on immune effector cells. However, much less is known about the effects of miRNAs on the susceptibility of target cells during T cell - target cell interaction. The goal of this project is to identify miRNAs affecting tumor cell recognition and killing by cognate cytotoxic T cells (CTLs). Therefore, the murine melanoma cell line B16F10 was transfected with a comprehensive

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murine miRNA library, followed by co-cultivation with a stable $CD8^+$ CTL line specific for the tumor antigen TRP-2. Individual miRNAs modulating the susceptibility to CTL mediated killing were identified in a luciferase based high throughput cytotoxicity assay system. The hits obtained with the screen were preselected and after several steps of validation, the strongest candidates were selected. Using databases and prediction algorithms, the target mRNAs of these miRNAs are being investigated and they will be confirmed experimentally. The role of the identified target genes in CTL mediated killing will be validated in functional assays including IFNγ ELISpot-assays and the impedance based xCELLigence system. Moreover, the immune-modulatory effects of the identified miRNAs will be confirmed *in vivo*. These miRNA species are of potential clinical interest, as they could help to improve both classical chemotherapy approaches and novel immunotherapeutic treatments against cancer, through the inclusion of, for instance, sensitizing miRNAs that make cancer cells weaker when facing the immune system.

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Manipulation of potassium channels in tumor specific T cells for improved functionality in the suppressive tumor microenvironment

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Background: Treatment of cancer with T cell therapy has shown promising results. However, T cells are not always able to fully control and eliminate the cancer, and this is likely due to a number of tumor suppressor mechanisms. We believe that the expression of matrix metalloproteinase 23 (MMP-23) may very well be a novel mechanism by which tumors suppress anti-tumor T cell responses. It has been shown that MMP-23 can bind and block the voltage-gated potassium channel Kv1.3. Together with Kca3.1, Kv1.3 is important for Ca^{2+} homeostasis during activation of T cells, and synthetic blockers of Kv1.3 and Kca3.1 has previously been shown to suppress T cell functionality. By blocking of Kv1.3, MMP-23 may therefore also be able to inhibit T cell activation, and a correlation has already been observed between expression of MMP-23 in melanoma and a poor response to immunotherapy as well as fewer tumor infiltrating lymphocytes.
Aim: We hope to highlight a new mechanism by which tumor cells suppress T cells; namely through blocking of Kv1.3 on T cells by the expression of MMP-23.
Methods: We have used CRISPR/cas9 and lentiviral transduction to genetically engineer T cells and cancer cell lines. In addition, ELISA and flow cytometry has been used to measure T cell cytokine production and proliferation, respectively.
Results: We have already shown inhibition of T cell proliferation and IFNγ production in the presence of synthetic blockers of Kv1.3 and Kca3.1. We have also created MMP-23 KO cancer cell lines, as well as introduced over-expression of MMP-23 in several cancer cell lines. Furthermore, we have genetically engineered T cells to over-express Kca3.1.
Conclusion: In the present study, we have shown inhibition of T cell activation when potassium channels Kv1.3 and Kca3.1 are blocked. We hope to show that tumors utilize this mechanism to suppress anti-tumor T cell responses by expressing MMP-23. Furthermore, we hope to find a way to circumvent this inhibition by genetically changing the potassium channels of the T cells, which may provide important knowledge to future T cell therapies against cancer.

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Evidence for T-cell mediated immunity of HPV vaccination by 3-colour fluorescent EliSpot assay

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Vaccination against Human Papillomaviruses (HPV) is recommended for girls from nine to fourteen years of age in Germany. The applied HPV vaccines consist of L1 virus-like particles (VLPs). The aim of this project was to evaluate the T-cell mediated immunity against L1 VLPs of the HPV-Types 6, 11, 16, and 18 in the fluorescence Enzyme Linked ImmunoSpot (EliSpot) Assay. We wanted to verify if, and in which manner, a T-cell mediated immunity could be monitored after vaccination based on the fluorescence-enabled detection of cytokine secretion patterns.
As a first step the experiment set-up was determined. Different incubation times from 24-72 h and antigen (VLPs) concentrations from different preparations were tested with vaccinated and unvaccinated donors to define parameters for the future application. These parameters were then verified by a higher number of vaccinated and unvaccinated donors. To evaluate T-cell mediated immunity, IFNy, IL-2 and IL-5 were detected simultaneously by establishment of a 3-colour fluorescence EliSpot assay.
Taken together, differentiation of the blood donor groups (fully vaccinated, i.e. freshly and long-term, partly vaccinated, and unvaccinated) was clearly possible by the fluorescence-based 3-colour EliSpot. We found with vaccinated individuals significantly higher T-cell reactivity with the used antigen preparations compared to non-vaccinated control donors. Characteristic cytokine secretion patterns for the vaccinated donors made it possible to distinguish (i) freshly vaccinated donors with high IFNy, medium IL-2, and high IL-5 secreting cell frequencies, (ii) long-term vaccinated donors with medium IFNy, high IL-2, and no IL-5 secreting T cell frequencies, and (iii) partly vaccinated donors with low IFNy and low IL-2 secretion.
Therefore, a characteristic pattern for immunogenicity after distinct vaccination schedules could be defined.
For the induction of an immunologic memory response by successful vaccination a T-cell based immune response is discussed to be necessary. The range of the cellular immune response for an effective vaccination should be confirmed by further investigations with more and well characterized blood donors. The aforementioned 3-color EliSpot fulfils all requirements for screening vaccinated and unvaccinated patients not only with healthy conditions but also with regard to montior effectiveness of other available, therapeutic vaccinations in patients with CINII or CINIII precancerous conditions. Furthermore these trials could facilitate the detection of significant differences between vaccinated and non vaccinated donors and open the possibility to correlate immunogenicity with effectiveness of vaccination.

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Upregulation of HLA class I expression, CIITA-related block of HLA class II expression and heterogenous expression of immune checkpoints in hepatocarcinomas: implications for new therapeutic approaches

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Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers and is one of the deadliest cancers ranking sixth in global incidence and second in terms of cancer deaths worldwide. Despite

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important advances in the HCC diagnosis and treatment in the recent decades, the 5-year survival rate remains dismal, (less than 10%), because of the high frequency of intrahepatic recurrence after hepatectomy and the low effect of systemic therapy. Thus novel complementary strategies of treatment are urgently required for this deadly disease. The recent revitalized enthusiasm for immunotherapeutic approaches to fight cancer has been fueled by the success of anti-checkpoint inhibitors and novel therapeutic vaccines based on tumor-specific peptides isolated from HLA molecules expressed in tumor cells.
Considering that immunotherapeutic strategies will best take advantage from combined approaches considering both the optimal triggering and the persistence of anti-tumor immune response, here we have investigated in detail the expression both of HLA molecules (HLA class I and class II) and immune checkpoints markers (PD-1 and PD-L1) in HCC tissue samples from 43 distinct tumors and a series of HCC cell lines.While normal hepatocytes displayed no HLA class I and HLA class II expression, HCC cells strongly upregulated HLA class I while remaining negative for HLA class II expression. HCC tumor tissues analyzed presented distinct degree of lymphocyte infiltration which, however did not correlate with the pre-existing diagnosis of HBV or HCV infection. Clear expression of PD-1 was detected in infiltrating cells, interspersed within the HCC tumor tissue and mainly represented by lymphocytes. In HCC tumor tissues with absent or moderate leukocyte infiltration, PD-L1 expression, when present, was observed in CD68-positive cells with monocyte-macrophage morphology. These cells were localized at the margin of tumor cell areas and usually were not infiltrating the tumor mass. In no instance did we find HCC tumor cells positive for PD-L1 expression. Interestingly, the absence of HLA class II expression in HCC cells correlated with lack of expression of the major HLA class II transactivator, CIITA, which could not be rescued even after treatment with its major inducer interferon-gamma. Taken together, our results support the idea that HCC cells, in contrast to other tumor types, by upregulating their HLA class I may indeed favor the display and presentation of possible tumor antigens to relevant tumor-specific cytolytic T lymphocytes, although they cannot act as surrogate antigen-presenting cells for HLA class II-restricted tumor antigens. The variability of immune checkpoint expression and particularly PD-L1 expression in infiltrating cells but not in tumor cells, should also be taken into account when envisaging strategies of anti-checkpoint inhibitors treatment in order to selected the appropriate HCC that express high levels of PD-1/PD-L1 markers.

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Live-cell assays for immune cell killing of 3D tumour spheroids
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Immunotherapies such as checkpoint inhibitors, CAR-Ts and immune-targeting Abs have great promise for cancer treatment. Tumour spheroid models potentially offer more translational biological insight than 2D cell models and more relevant analysis of cancer immunotherapy agents in vitro. Here we describe novel image-based, immune cell-killing assays of 3D tumour spheroids based on IncuCyte live-cell analysis.
Single spheroids were formed from human tumour cell lines expressing RFP in 96-well ULA plates. Immune cells were then added and spheroid viability was assessed over time (up to 10 days) by measuring the loss of RFP fluorescence. This method is exemplified with a range of immune cell activators, stimulating T cell (anti-CD3 and IL-2) or natural killer cell (IL-12 & IL-2) PBMC sub-populations. As expected the magnitude and rate of cytotoxicity was effector-to-target cell ratio dependent. In an antibody-dependent cell-mediated cytotoxicity (ADCC) format, Herceptin induced a concentration-dependent specific killing of Her-2 expressing tumour cells (SKOV-3). Higher concentrations of Herceptin were required in 3D vs 2D ADCC assays.
These data demonstrate the capability to kinetically visualise and quantify 3D immune cell killing and ADCC assays, and illustrates how these assays can be extended from traditional 2D cultures to 3D.

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These assays will be highly valuable in the search for novel immuno-modulators.

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The Role of RAF-MAPK signalling in Dendritic cells

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Mitogen Activated Protein Kinases (MAPKs) are a family of serine/threonine protein kinases that regulate fundamental cellular processes like cell division, migration, differentiation and death. The RAS oncogene, which is mutated in 30% of human cancers, activates the highly conserved RAF-MEK1/2-ERK1/2 (MAPK) cascade and many anti-tumor drugs with clinical success have targeted the components of this pathway. While the role of MAPKs in controlling tumor cell proliferation, survival and metastasis is well studied, relatively little is known on the contribution of these MAPKs in regulating immune responses. Previous studies have shown that treatment of DCs with MEK1 inhibitor Trametinib enhanced DC activation. We detect that treating DCs with tumor inhibiting concentrations of RAF and MEK inhibitors differentially regulates DC maturation and activation, showing that inhibition of MEK1/2 but not RAF kinases significantly enhances DC maturation and activation in DCs derived from both mice and human. In contrast to the effect of MEK1/2 inhibition, the blockade of RAF kinases even inhibited LPS-induced stimulation of DCs. These data suggest that RAF kinases and MEK1/2 may have independent roles in the regulation of DC function, suggesting that MAPK signalling is probably not linear as in other cell types. Further, we detect that the proteostasis of RAF kinases are altered during DC maturation and that these Serine Threonine kinases are activated in DCs. Improved understanding of how MAPKs are involved in the induction and modulation of immune responses would enable us to adroitly administer novel therapeutics for a long-lasting anti-tumor response.

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The anatomical location determines type of lymphocyte infiltration in tumors of same etiology

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The immune contexture of a tumor correlates significantly with clinical outcome. While intimate tumor-host interactions are known to shape the tumor's immune microenvironment, the influence of the anatomical location in which the tumor arises remains largely unknown since this influence is easily obscured by the different, and sometimes immune modulating, oncogenic pathways that can be in play in one type of tumor.
Therefore, we studied the lymphocytes in a series of tumors, metastatic lymph nodes (LN) and peripheral blood samples of patients with human papillomavirus (HPV)-induced primary cervical carcinoma (CxCa) and oropharyngeal squamous cell carcinoma (OPSCC), two types of tumors arising in distinct anatomical locations but sharing the same etiology. We performed an in-depth analysis of the lymphocytic infiltrates in the tumor microenvironment (TME) using 36-parameter mass cytometry

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(CyTOF) analysis and results were compared to metastatic CxCa LN and matched peripheral blood mononuclear cell (PBMC) samples. The tumor and LN cell suspensions were prepared by enzymatic and/or mechanistic dissociation and cryopreserved until CyTOF analysis. HPV status of the tumors was determined by GP5+/6+ PCR and p16 immunohistochemistry staining. HPV16 E6/E7-specific T cell reactivity within HPV16+ tumors and LN was determined by proliferation assay, cytokine production and intracellular cytokine staining.
Clear phenotypic differences between immune cells infiltrating the TME of CxCa and OPSCC tumors were found. Whereas HPV+ OPSCC tumors were strongly infiltrated with IgM+, non-class switched B cells and CD4+ T cells, HPV+ CxCa tumors were strongly infiltrated with CD8+ T cells. The CD4:CD8 ratio was 2.5x higher in OPSCC than CxCa and both T cell subset frequencies were close to those found in the tissue of origin. PBMC analysis revealed a high level of comparability between CxCa and OPSCC patients. Subsequent unsupervised hierarchical clustering through the CITRUS algorithm led to the identification of distinctive tumor-specific populations of CD161+ effector memory CD4+ T cells and CD103+ tissue-resident effector CD8+ T cells, both with a highly activated CD38+, HLA-DR+, and/or PD-1+ phenotype. CD161+ CD4+ T cells produced the highest cytokine levels and their numbers correlated with the detection of intratumoral HPV-specific CD4+ T cells. Interestingly, CxCa were often less infiltrated with HPV-specific CD4+ T cells despite their presence in metastatic CxCa lymph nodes. The differences in CD4+ T-cell infiltration (total and HPV-specific) may explain why (HPV-specific) CD4+ T cells have an impact on survival in OPSCC but not CxCa.
In conclusion, the anatomical location has an impact on a tumor's immune contexture and this bears consequences on clinical outcome.

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Oncogenic BRAFV600E governs regulatory T cell recruitment during melanoma tumorigenesis
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Regulatory T cells (Tregs) are critical mediators of immune suppression in established tumors, although little is known about their role in restraining immune surveillance during tumorigenesis. Here an inducible autochthonous melanoma model was employed to investigate the earliest Treg and CD8 effector T cell responses during oncogene-driven tumorigenesis. Induction of oncogenic $BRAF^{V600E}$ and loss of Pten in melanocytes led to the localized accumulation of $FoxP3^{+}$ Tregs, but not CD8 T cells, within 1 week of detectable increases in melanocyte differentiation antigen expression. Melanoma tumorigenesis elicited an early expansion of shared tumor/self antigen-specific, thymically derived Tregs in draining lymph nodes, and induced their subsequent recruitment to sites of tumorigenesis in the skin. Lymph node egress of tumor-activated Tregs was required for their C-C chemokine receptor 4 (Ccr4) dependent homing to nascent tumor sites. Notably, $BRAF^{V600E}$ signaling controlled the expression of Ccr4-cognate chemokines, and governed the recruitment of Tregs to tumor-induced skin sites. $BRAF^{V600E}$ expression alone in melanocytes resulted in nevus formation and associated Treg recruitment, indicating that $BRAF^{V600E}$ signaling is sufficient to recruit Tregs. Treg depletion liberated immunosurveillance, evidenced by CD8 T cell responses against the tumor/self-antigen gp100, which was concurrent with the formation of microscopic neoplasia. These studies establish a novel role for $BRAF^{V600E}$ as a tumor cell-intrinsic mediator of immune evasion, and underscore the critical early role of Treg-mediated suppression during autochthonous tumorigenesis.

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Pan-cancer analysis of human endogenous retrovirus expression reveals markers for prognosis and immunotherapy response in clear cell renal carcinoma

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Background: There is substantial interest in the identification of antigens that can drive anti-tumor immune responses, both to discover improved biomarkers of response to immunotherapy and provide novel immunotherapy targets for translational development. One group of tumor antigens that has been understudied to date is the set of human endogenous retroviruses (hERVs).
Methods: In the current study, we applied a novel software tool (hervQuant) developed in our lab to quantify the expression of over 3,000 intact hERV sequences from The Cancer Genome Atlas RNA-seq, Kidney Renal Clear Cell (KIRC) Ribo-seq, and anti-PD-1-treated KIRC RNA-seq datasets. We comprehensively evaluated the role of hERVs in tumor/immune interactions across the included tumor types, focusing in on KIRC for the majority of our study.
Results: hERV expression was associated with clinical prognosis in several tumor types, most significantly in TCGA KIRC. We explored two mechanisms by which hERV expression may influence the tumor-immune microenvironment in KIRC: 1) RIG-I-like signaling, and 2) hERV antigen activation of adaptive immunity. We observed two distinct classes of hERVs with positive (*RIG-I-like up*) and negative (*RIG-I-like down*) correlation patterns to genes associated with RIG-I-like-mediated NF-kB inflammation, as well as a class of hERVs with strong positive correlation with distinct B-cell clones (*B-cell correlated*). Using these signatures, we show the ability of hERV expression to provide prognostic value independent of clinical staging and molecular subtyping (multivariable Cox regression hazard ratios: *RIG-I-like up*: 0.69, *RIG-I-like down*: 3.03, *B-cell correlated*: 0.29). Lastly, we predicted and validated the antigenicity of specific hERV epitopes using Ribo-seq data (to confirm translation) and generation of peptide-bound HLA (to confirm MHC presentation of epitopes, exchange efficiencies 16 - 73%). We demonstrate these hERV epitopes are significantly more highly expressed in biopsy samples from anti-PD-1-responsive KIRC patient samples compared to non-responsive tumors (Mann-Whitney range for hERV qPCR and hervQuant p-values: 0.0348 - 0.0028) suggesting these epitopes may serve as novel biomarkers and potential therapeutic targets in the context of PD-1 axis inhibition.
Conclusions: We show via integrated survival modeling that hERV signatures carry more prognostic information than published molecular subtyping and nearly as much information as traditional clinical staging. Clinically, a hERV-based signature for prognosis could dramatically improve our ability to predict for patient survival. Additionally, hERV expression is increased in primary clinical response versus resistance to PD-1 axis inhibition in KIRC. These findings suggest that the expression of tumor-specific hERVs may be used to predict for patient responsiveness to immunotherapy, and more broadly that these hERVs may serve as novel and non-personalized tumor vaccine targets.

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Omental fat-induced immune alterations during metastatic spreading of epithelial ovarian cancer: insights from tissue explant culture

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The density of tumor-infiltrating lymphocytes (TILs), especially intraepithelial TILs, is instrumental in

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predicting the clinical outcome in epithelial ovarian cancer, underlying the importance of the anti-tumor immune response in disease control. Because these tumors most frequently spread along the omentum, close interactions between tumors and fat occur in late stages of the disease. However, little is known about how this might impact the immune landscape.
To address that question, we used a cohort of ovarian cancer specimens for histological analysis, cytokine and metabolic profiling. Furthermore, we developed an ovarian cancer tissue explant culture model and treated whole tissue explants with drugs before assessing immune cell density, distribution and activation status.
The presence of peritoneal fat inside the tumor is associated with massive tumor infiltration by T cells and macrophages. Fat-containing tumors display smoldering inflammation and low expression of markers of an ongoing anti-tumor cytotoxic response. TILs are located away from tumor cells, accumulate around adipocyte-rich areas and produce high amounts of the tumor-supporting chemokine CCL5.
Inhibition of the CCL5-CCR5 axis in whole-tissue explants using Maraviroc effectively restores cytotoxic T cell distribution across the tissue by rerouting them towards other chemokines. In addition, Maraviroc treatment triggers a Th1-like cytokine polarization, as well as a metabolic switch, in macrophages in a fat-dependent fashion. Finally, fatty acid import blockade in macrophages appears as an alternative strategy to reestablish (i) a Th1/cytotoxic cytokine profile and (ii) an appropriate T cell distribution. The underlying mechanisms are currently being examined.

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Characterization of rarely detectable tumor-associated antigens (TAA)-specific CD8+ T cells in HCC patients

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Hepatocellular carcinoma (HCC) is the second-leading cause of cancer-related deaths worldwide with limited therapeutic options. Since CD8+ T-cell responses targeting tumor associated antigens (TAA) beneficially influence patients` survival and checkpoint blockade showed promising success in various cancers, immunotherapy based on TAA-specific CD8+ T cells is considered to be a promising approach. However, determinants of TAA-specific T-cell responses are largely unknown. Therefore, we characterized TAA-specific CD8+ T cells in detail by using a tetramer-based enrichment strategy to assess their immunotherapeutic potential.
The phenotype of circulating TAA-specific CD8+ T cells derived from the peripheral blood of HCC patients at baseline time points (n=43), patients with liver cirrhosis (n=29), melanoma patients (n=19) and healthy donors (HD) (n=26) were analyzed by multicolour flow cytometry after peptide/MHC class I-tetramer-based magnetic bead enrichment. For this, peptide/HLA-A*02-tetramers loaded with NY-ESO-$1_{157-165}$, Melan-A/Mart-1_{26-35} and MAGE-$A3_{271-279}$ or peptide/HLA-A*03-tetramers loaded with MAGE-$A1_{96-104}$ were used. In addition, corresponding antigen levels of NY-ESO-1, MAGE-A3 and MAGE-A1 in serum were analyzed by ELISA.
Our data revealed no difference in the low frequency of TAA-specific CD8+ T cells detectable between HCC patients and control cohorts. Interestingly, only a subset of TAA-specific CD8+ T cells exhibited an antigen-experienced phenotype in patients suffering from liver cirrhosis and HCC. Of note, while MAGE-A3 was not detectable with the applied method, serum levels of MAGE-A1 were significantly higher in HCC patients compared to HD but were comparable to patients with liver cirrhosis. However, MAGE-A1 serum level did not correlate with frequency of MAGE-specific CD8+ T cells or percent of antigen-experienced MAGE-specific CD8+ T cells. The few antigen-experienced MAGE-specific CD8+ T cells that could be detected in HCC patients expressed high levels of CD127 and TCF1 reflecting high potential of homeostatic proliferation and only a minor fraction exhibited PD-1 expression. Importantly, in addition to the minor PD-1 expression, antigen-experienced MAGE-specific CD8+ T cells lacked the $Eomes^{hi}$ $Tbet^{dim}$ signature of exhausted T cells.

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In sum, our data suggest that T cell exhaustion plays a minor role in T cell failure of circulating TAA-specific CD8+ T cells in HCC patients, at least for those specifies analyzed in this study. Thus, a prime/boost strategy might be required to induce MAGE-specific CD8+ T cells in MAGE positive HCC patients.

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Increased replication and spread of the oncolytic VSV-GP in a syngeneic prostate cancer model does not correlate with improved therapeutic outcome

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The cure of Prostate Cancer when it progresses after conventional therapy is still an unmet medical need. Oncolytic viruses (OVs) that preferentially replicate in and kill tumor cells are an innovative treatment option for cancer patients after failure of common therapeutic strategies. OVs cause cell lysis, which can also lead to release of tumor antigens. Paired with the immune-stimulating action the OV, this can ultimately trigger a strong anti-tumor immune response. Here we examine the use of VSV-GP, a novel and potent oncolytic virus (OV) based on the vesicular stomatitis virus and pseudotyped with the lymphocytic choriomeningitis virus glycoprotein, for the treatment of PCa and address potential resistance mechanisms.
We tested the efficacy of VSV-GP in both xenograft and syngeneic prostate cancer mouse models. We have observed high response rates in two different subcutaneous xenograft models, both after local and systemic administration of VSV-GP. Furthermore, a single intravenous administration of VSV-GP in a bone metastasis prostate cancer model was sufficient to cause tumor remission in all treated mice. In the TRAMP-C1 subcutaneous syngeneic model, intratumoral treatment with VSV-GP resulted in tumor growth delay and significant increase of the median survival, though with little effect on overall survival. We hypothesized that the IFN-I response in TRAMP-C1 cells was the main factor limiting the OV effect. Inhibition or knock-out of the IFN-I response in the tumor enhanced replication of VSV-GP and prolonged its persistence but, surprisingly, did not improve the therapeutic outcome. We are currently performing expressome analysis of TRAMP-C1-IFNAR$^{-/-}$ tumors and their wild-type counterparts to identify further resistance mechanisms.
In conclusion, VSV-GP is a promising novel therapeutic for the treatment of prostate cancer. To optimize the efficiency of VSV-GP, further studies will be necessary to better understand how the oncolytic effect, the IFN-I response and anti-tumor immune response interact in the OV scenario. The TRAMP-C1-IFNAR$^{-/-}$ model is an ideal tool to screen for further resistance mechanisms and identify what strategies will result in enhanced therapeutic outcome.

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Myeloid derived suppressor cells (MDSC) determine outcome in ovarian cancer

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Substantial evidence about the importance of the immune system in the battle against cancer, has led to an increased interest in developing immunotherapies. However, for epithelial ovarian cancer, clinical trials show poor response rates. Immune escape via the presence of immune suppressive cells, including myeloid derived suppressor cells (MDSC) in the tumor microenvironment can explain this relative failure. Previously, our lab has demonstrated that upon macrophage depletion with Clodronate lyposomes, mice died faster ($p< 0,01$) in comparison to controls and showed an influx of monocytic MDSC ($p=0,004$). Also, in ovarian cancer patients, an increase in monocytic MDSC (mMDSC) resulted in reduced survival.
To determine the suppressive capacity of MDSC, *in vitro* co-culture studies were performed with $CD8^+$ cells (isolated from the spleen of naïve C57BL/6 mice) and MDSC, originating from either bone marrow of naïve mice, or stimulated for six hours with ID8-fLuc culture supernatants or ascites from ID8 tumor bearing mice. In a next experiment, immune changes were measured over time in tumorbearing ID8 mice (n=20). Five mice were sacrificed respectively one, three, five and ten weeks after tumor inoculation. Ascites and serum were collected to assess the immune system with fluorescent activated cell sorting (FACS) and cytometric bead array (CBA).
In vitro, activated MDSC were able to stop $CD8^+$ T cell proliferation and also reduced the total number of $CD8^+$ T cells. *In vivo*, a significant increase of mMDSC (p-adjusted = 0,02) and a similar trend is seen for M2 macrophages (p-adjusted = 0,07) in ascites at week 10 was present, revealing the high immune suppressive environment. In addition, an increase in MIP-1b was present in serum of mice at week 10 after tumor inoculation compared to week 1 (Mann-Whitney U, p = 0,02).
We can conclude that both our human and murine data at this moment point towards an important role of the innate immune system (represented mainly by MDSC and M2 macrophages) in the immunosuppression of ovarian cancer.

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Molecular mechanisms responsible for CCR5 regulation on MDSC in melanoma

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Melanoma microenvironment is characterized by a strong immunosuppressive network, where myeloid-derived suppressor cells (MDSC) play a major role. MDSC represent a heterogeneous population of myeloid cells that fail to differentiate into granulocytes, macrophages or dendritic cells. They were shown to inhibit anti-tumor activity of T and NK cells and stimulate regulatory T cells during tumor progression. MDSC migrate and accumulate in the tumor microenvironment due to the interactions between chemokine receptors and their ligands produced by tumor and stroma cells. We found previously a significant accumulation of MDSC expressing chemokine receptor CCR5 in skin melanoma lesions and metastatic lymph nodes as compared to the peripheral blood and the bone marrow of melanoma-bearing *RET* transgenic mice. This enrichment was associated with increased concentrations of CCR5 ligands and tumor progression. Importantly, tumor-infiltrating $CCR5^+$ MDSC displayed higher immunosuppressive activity than their $CCR5^-$ counterparts. Blocking CCR5/CCR5 ligand interactions increased survival of tumor-bearing mice associated with a reduced migration and immunosuppressive potential in tumor lesions. In melanoma patients, $CCR5^+$ MDSC were enriched at the tumor site that was correlated with enhanced production of CCR5 ligands. Furthermore, $CCR5^+$ MDSC were also enriched in the blood of melanoma patients compared to healthy donors and showed higher production of immunosuppressive molecules than their $CCR5^-$ counterparts.
Here we are deciphering the molecular mechanisms of CCR5 upregulation on MDSC leading not only to their recruitment into melanoma lesions but also to stimulation of their immunosuppressive activity. Studying the effect of cytokine, chemokine and TLR ligand-induced signaling as well as tumor-derived extracellular vesicles on CCR5 expression, we found a significant upregulation of CCR5 on immature

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myeloid cells mediated by TLR2 stimulation. In addition, the *in vitro* differentiation of MDSC from immature myeloid cells by IL-6 and GM-CSF was accompanied by CCR5 upregulation. Interestingly, $CCR5^+$ tumor-infiltrating MDSC showed increased levels of STAT3 phosphorylation compared to their $CCR5^-$ counterparts.
Altogether, our findings define a critical role for CCR5 in the recruitment and activation of MDSC. We suggest that the targeting of CCR5-positive MDSC could represent a novel strategy for melanoma treatment.

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Tumor microenvironment T-cell repertoire and mutational load are independently associated with the outcome of sequential checkpoint inhibition in melanoma

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Purpose: To understand prognostic factors for outcome between differentially sequenced nivolumab and ipilimumab in a randomized phase II trial, we measured T-cell infiltration and PD-L1 by immunohistochemistry, T-cell repertoire metrics and mutational load within the tumor using next-generation sequencing (NGS) and assessed association of those parameters with response and overall survival.
Patients and methods: Immunosequencing of the T-cell receptor beta chain locus (TCRb) from DNA of 91 pre-treatment tumor samples and an additional 22 pairs of matched pre- and post-treatment samples from a randomized trial of nivolumab followed by ipilimumab (nivo/ipi), or the reverse (ipi/nivo) was performed to measure T-cell clonality and fraction. Mutational and neoantigen load were also assessed by NGS in 82 of the 91 patients. Tumors were stained using immunohistochemistry for PD-L1 and $CD8^+$ T-cells.
Results: Pre-treatment tumor TCR clonality, mutational and neoantigen load were marginally associated with best response with nivo/ipi (P = 0.04, 0.06 and 0.05, respectively), but not ipi/nivo. Amalgamated pre-treatment mutational load and tumor T-cell fraction were significantly associated with best response with nivo/ipi (P = .002). Pre-treatment PD-L1 staining intensity and $CD8^+$ T-cell counts were correlated with T-cell fraction and clonality, but not mutational or neoantigen load. Patients with increased T-cell fraction post-treatment at week 13 had a 30-fold increased likelihood of survival (P = .002).
Conclusion: Mutational and neoantigen load, and T-cell infiltrate within the tumor, were associated with outcome with sequential checkpoint inhibition using nivolumab then ipilimumab, but not when ipilimumab was administered before nivolumab.

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History of prior immunotherapy changes relationship between tumor mutations and TIL repertoire

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Background: Recent efforts to identify tumor microenvironment (TME) features predictive of response to immunotherapy have focused on understanding the relationship between tumor mutations and tumor infiltrating lymphocytes (TILs). The underlying hypothesis is that mutations give rise to neoantigens and a subset of neoantigens provide clinically efficacious targets for TILs.
Aims: Here, we re-examine whole exome (WES) and immunosequencing data from a recently published cohort of melanoma patients (https://github.com/riazn/bms038_analysis)[1], with an emphasis on a) identifying pre-treatment TME biomarkers predictive of response to nivolumab and b) understanding how prior immunotherapy changes the relationship between tumor mutations and the TIL repertoire.
Results: Across all subjects (n = 42), filtering tumor mutations with NetMHC, which identifies putative neoantigens, provides objectively better predictions of disease progression than counting total mutations (average AUC ROC = 0.70 & 0.62, respectively). In addition, total neoantigens provide consistent predictive power, regardless of prior therapy with ipilimumab (AUC ROC = 0.69 & 0.70, without & with prior ipi), compared to total mutations (AUC ROC = 0.64 & 0.58, without & with prior ipi). Median neoantigen abundance weakly correlates with T-cell repertoire clonality (Spearman's Rho = 0.35, p = 0.05) and, in patients without prior ipi therapy, total neoantigens and TIL CD4:CD8 ratio also correlate with clonality (Spearman's Rho = -0.44 & -0.48, p = 0.09 & 0.06, respectively). The frequency for each mutation enabled estimation of neoantigen density or neoantigens per cell, which may add predictive capability (NS) of WES and be informative for studying the evolution of the TME in patients with prior ipi therapy.
Conclusions: While total neoantigens appears to be a reliable predictor of response, the relationship between tumor mutations/neoantigens and the TIL repertoire is clearly altered by a history of prior immunotherapy. How the presence of key driver mutations such as RAS and BRAF, relative to wildtype, influences this relationship will be of future interest.
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The CliniMACS Prodigy® and CliniMACS® Electroporator platforms enable automated and GMP-compliant production of dendritic cell vaccines

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The efficacy and safety of dendritic cell (DC) vaccines in cancer immunotherapy have been proven in several clinical studies using monocyte-derived dendritic cells (Mo-DC), plasmacytoid DC (PDC) or CD1c+ myeloid DC (MDC) loaded with tumor derived peptides, resulting in successful induction of anti-tumor immune response and improved overall survival. Vaccines consisting of both PDC and MDC are currently been tested in a phase III melanoma study (Radboud University Medical Center, M. Jolanda de Vries). Recently the natural cross-priming capacity of the CD141+ CLEC9A+ DC subset (XP-DC) has been shown to be essential for the initiation of effective cytotoxic T cell (CTL) responses against tumors, thus providing a promising vaccination approach in cancer immunotherapy.
The preparation of DC products for clinical settings requires several steps including cell washing, cell separation, cell cultivation, peptide loading, cell activation, electroporation and cell formulation. To facilitate the regulatory requirements for cell-based therapeutics we have integrated all manufacturing steps in closed systems operated by an automated cell-processing instrument, the CliniMACS Prodigy® and an automated electroporation instrument, the CliniMACS® Electroporator. In order to address the need of various DC vaccines we developed specific CliniMACS Prodigy Systems for the manufacturing of Mo-DC, PDC, MDC, XP-DC, and PDC combined with MDC. To complete our DC platform we also plan to provide a process that allows the enrichment and formulation of blood PanDCs, which will include PDC, MDC and XP-DC. To simplify quality and in-process control we optimized a 7-color staining protocol, and a tool for automated data acquisition and analysis (ExpressMode) for the MACSQuant Analyzer, which discriminates between the very rare XP-DC, PDC and MDC.
Here we give an overview of Miltenyi Biotec´s instrument platforms and diverse processes for the automated manufacture of dendritic cell vaccines, enabling reproducible and automated DC enrichment, peptide loading, activation and functional tuning by efficient electroporation in a closed system that facilitates compliance with GMP requirements.

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The recognition patterns of T cell receptors identified using peptide-MHC multimers labeled with DNA barcodes

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The promiscuous nature of T-cell receptors (TCRs) is fundamental for our ability to recognize and fight a large range of pathogens. However, this promiscuity also enhances the risk of chronic inflammation and immunopathology due to cross-recognition of foreign and self-antigens, and makes it challenging to fully understand and control T-cell recognition.
Existing technologies provide limited information about the key requirements for T-cell recognition and the ability of TCRs to cross-recognize structurally related elements. Herein we present a proof-of-concept of a novel strategy to establish TCR recognition patterns that will contribute to our fundamental understanding of TCR interactions with the peptide-major histocompatibility complex (pMHC) and can serve as an important tool to assess potential TCR cross-recognition prior to clinical development. We examined the requirements for TCR interaction with pMHC by the use of libraries of peptide variants, which are generated from sequentially substituting each position of the originally identified target peptide with all naturally occurring amino acids. We assembled these onto MHC multimers labeled with DNA barcodes, which allows the simultaneous assessment of the interaction between a single TCR and multiple peptide variations. With this approach, we established an affinity-based hierarchy of the pMHC interactions, which enabled us to describe the TCR recognition motif, here termed the TCR fingerprint. We identified the fingerprint of two TCRs isolated from Merkel cell carcinoma (MCC) patients that recognize Merkel Cell Polyomavirus (MCP*y*V)-derived Large T Antigen (LTA) peptides restricted to HLA-B*0702 and HLA-A*2402, respectively. For these TCRs, only a few interaction points were required to enable the recognition of pMHC, and the essential amino acids at these critical positions could be determined, which was not possible using standard alanine substitution libraries. Analyzing a set of 12 different T-cell clones that all recognize the same MCP*y*V-derived HLA-A*0201-restricted peptide, we found substantial differences between these TCRs' recognition profiles. Based on their TCR fingerprint, the T-cell clones clustered independently on their functional interaction strength (EC_{50}) and the patient origin. Moreover, the required number of interaction points was inversely correlated to functional efficacy, indicating that high-affinity TCRs may possess a higher risk for cross-recognition and that the TCR fingerprints may be predictive for both the functional capacity and the cross-recognition potential of TCRs. Among these TCRs that recognize the same pMHC targets, the TCR fingerprint enabled us to identify cross-reactive peptides, demonstrating the value of this strategy for the screening and selection of TCRs intended for clinical applications.

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LP-141 CliniMACS Prodigy System: primary cross-presenting DCs for the generation of cancer vaccines

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In mouse, CD8-alpha$^+$ dendritic cells (DCs) represent a subpopulation that excels in cross-presentation and is essential for initiating effective cytotoxic T cell (CTL) responses against tumors. An equivalent human subset of professional cross-priming DCs (XP-DCs) has recently been defined as lineage negative, MHC II^{hi}, CD141$^+$, XCR-1$^+$, CLEC9A$^+$.
Here we demonstrate that XP-DCs isolated using a fully automated clinical grade process acquire fully mature phenotype and produce pro-inflammatory cytokines upon stimulation with TLR3 agonists. Furthermore, isolated XP-DCs take up, process and present antigens and are thereby able to stimulate T-cells in an antigen-specific manner.
To test their stimulatory capacity XP-DCs were isolated from CMV-seropositive donors in a two-step procedure using the CliniMACS Prodigy instrument. Enriched XP-DCs were loaded with CMV-derived peptides and simultaneously activated by the means of TLR3-agonist poly I:C. Activated and antigen loaded XP-DCs were then co-cultured with autologous CD8$^+$ T cells. Antigen specific T cell activation was assessed based on cell proliferation and pp65-Tetramer staining. Additionally, IFNgamma and TNFalpha production in restimulated CTLs was probed by intracellular staining. To enable a fully GMP compliant process Bo-112, a GMP-grade TLR-3 ligand, was tested as a stimulus and induced production of the same specific set of cytokines as poly I:C.

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XP-DCs upregulated activation markers and produced a specific set of cytokines including vast amounts of IFN-lambda upon stimulation with TLR3 agonists. T cells stimulated by activated and antigen-loaded XP-DCs proliferated in an antigen specific manner as shown by increasing frequency and cell numbers of tetramer positive CTLs. Antigen specificity of the expanded cells was further confirmed by intracellular cytokine staining upon restimulation with CMV-derived antigens.
The scarcity of XP-DCs is a major limitation in utilizing these cells for clinical applications. Here we show that isolation of up to 10 million XP-DCs from leukapheresis products using the CliniMACS Prodigy might be feasible. On the other hand previous data indicate that Flt3L mobilization substantially increases the frequency of all DC subsets in the circulation including XP-DCs. Combination of the Flt3L treatment with the fully automated separation could therefore accelerate clinical utilization of XP-DCs.

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Automated cell processing and electroporation for generation and genetic engineering of monocyte-derived dendritic cells

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The value and safety of dendritic cell (DC) vaccines in cancer immunotherapy have been proven in several clinical studies using monocyte-derived dendritic cells (Mo-DCs) over the last two decades. However, the high diversity in maturation and antigen-loading protocols results in cellular products with different therapeutic efficacy. Moreover, the immune status of the patient may additionally impact the function of the resulting DC-vaccine. In this context genetic modification revealed to be a potent tool in enhancing the functionality of cellular products.
Standardized and reproducible GMP-compliant manufacturing of such cellular products, however, requires instrumentation with specific characteristics that are not always addressed by conventional technologies.
Recently, we have presented the CliniMACS Prodigy®, a new platform integrating all manufacturing steps in a closed system, which enables reproducible and fast production of Mo-DC vaccines for clinical use. CliniMACS® Electroporator represents a further development of this platform, which now also enables automated electroporation of Mo-DCs in a closed system.
Here, we generated Mo-DCs using the CliniMACS Prodigy and electroporated them with different mRNAs using the CliniMACS Electroporator. We optimized transfection conditions and electroporation protocols with GFP mRNA as a proof of concept. Using mRNA encoding immune relevant antigens we generated Mo-DCs that stimulated autologous, antigen specific, cytotoxic T cells with comparable efficacy as Mo-DCs that have been loaded extracellularly with the relevant peptide. We also introduced genes that may improve DC functions and proved their expression by both flow cytometry and functional assays.
In summary, we developed an automated, large scale, closed process that enables efficient transfection of Mo-DCs for clinical use enabling antigen delivery and functional tuning.

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Exploring human T lymphocytes redirected to the tumor neoantigen EGFRvIII for adoptive cellular immunotherapy of non-small cell lung cancer

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Adoptive cellular therapy (ACT) using genetically engineered T cells expressing a chimeric-antigen-receptor (CAR) to redirect their specificity holds great promise in tumor immunotherapy. However, in contrast to remarkable clinical responses observed with CD19-CAR T cells in B-cell leukemias, efficacy of ACT in solid tumors is often hampered by insufficient and non-durable immunity of T cells facing an immunosuppressive tumor microenvironment. Moreover, identifying tumor-specific neoantigens with low off-target activity is difficult. The epithelial growth factor variant III (EGFRvIII) is only found on neoplastic tissue and represents a promising candidate target for ACT in e.g. glioblastoma as already shown in clinical trials and non-small cell lung cancer (NSCLC). Since EGFRvIII-CAR T cells have not yet been tested in NSCLC, we therefore aimed in this preclinical study to evaluate antitumor immunity of EGFRvIII-CAR T cells against NSLCL in vitro and in vivo using a xenograft mouse model.
Human, preactivated T cells were retrovirally transduced, and CAR expression was analyzed by flow cytometry 7 days (d) post transduction. Stable EGFRvIII transfectants of a HLA class I/II-deficient melanoma cell line (Ma-Mel-86) or the NSLCL cell line A549 were used as targets. EGFRvIII-CAR T cells were examined for IFN-γ/granzyme B release and cytotoxicity using ELISpot-, 51Cr-release- and tumor-lymphocyte colony forming assays (T-LCFA). To evaluate the therapeutic potential in vivo, intratumoral T-cell transfer into NSG mice displaying palpable subcutaneous tumor engraftment was performed.
Upon retroviral transduction >85% T cells expressed the EGFRvIII-CAR as confirmed by flow cytometry. EGFRvIII-CAR T cells induced vigorous responses to both EGFRvIII+ tumor targets in vitro as measured by IFN-γ and granzyme B release and elicited potent cytotoxicity (>60%) upon short-term 5h coculture (51Cr-release) and >90% in T-LCFA after 2d. In vivo experiments evaluating the therapeutic potential of bulk EGFRvIII-CAR T cells with subsets displaying a stem cell and central memory phenotype using a NSG xenograft mouse model are currently in progress.
The tumor-specific neoantigen EGFRvIII represents a very attractive target for ACT. We could already prove effective reactivity of EGFRvIII-CAR T cells towards EGFRvIII-expressing melanoma and lung cancer cells in vitro. Although EGFRvIII appears not to be a dominant target among the different EGFR mutations found in NSCLC patients, EGFRvIII-CAR ACT might provide valuable information as a proof of concept study for immunotherapy in lung cancer and might contribute to improve ACT for solid tumors.

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The development of human γδ-T antigen presenting cells as a clinical grade cancer vaccine

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Introduction: Gamma-delta T Antigen Presenting Cells (γδT-APC) represent a novel type of therapeutic cellular vaccine with potential to mobilize effective anti-tumour immune responses in patients with cancer compared to existing approaches utilising dendritic cells (DC). Typically derived from peripheral monocytes (MoDc), dendritic cells are the prototypical antigen presenting cells used in many clinical trials to date. DCs, however, are limited by an inability to expand and variable recovery from cryopreservation. Conversely, T-cells can be expanded form relatively small blood volumes to provide multiple doses of cellular therapy and can be recovered from cryopreservation with relative ease. We have previously demonstrated in a pre-clinical system the potent antigen presentation ability of γδT-APC (Front Immunol. 2014 Jul 23;5:344) and here, we describe a manufacturing process compliant with current regulatory guidelines.
Methods: Up to 200 mL peripheral blood was taken from patients with either melanoma, primary liver

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cancer or healthy controls. Peripheral blood mononuclear cells were isolated and resuspended to 2 x 10^6 cells/mL in GMP grade culture medium (TexMACS, Miltenyi Biotec), supplemented with 1% pooled platelet lysate (Cook Regentec) and a generic γδT-cell antigen (zoledronic acid). After 48 hours, cells were stimulated with IL-2 and IL-15 (both 100 IU/mL, Miltenyi Biotec). All cultures were carried out in gas permeable culture bags or G-Rex culture devices (Wilson Wolf). In addition, after initiation of expansion, cells were transferred to a wave bioreactor (GE Healthcare, Xuri) and cultured for a further 5-9 days. Maintenance of APC function was confirmed in antigen presentation assays.
Results: The frequency of γδT-cells in peripheral blood varied from 0.2% - 6% of all T cells. Following expansion, γδT-cell frequency increased (mean 85%, range 55-93%) with viability of >90%. Expansion in G-Rex chambers was sufficient to yield 2 -3 x 10^9 cells whilst transfer into the wave bioreactor permitted expansion up to 20 - 30 x 10^9 cells. Following expansion, APC function was confirmed by induction of IFN-γ by a Flu-M1 specific CD8 reporter line in response to peptide or whole protein loaded γδT-APC.
Conclusion: We have demonstrated the expansion of γδT-APC in a GMP culture system. Following expansion, these cells can be recovered from cryopreservation and retain potent APC function. This data is being compiled into a submission to the regulatory authority to carry out a vaccination study in patients with solid tumours, refractory to standard of care therapy.

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In vitro generation of tumor antigen-specific T cells from patient and healthy donor stem cells

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Chimeric antigen receptor (CAR)-transduced peripheral blood lymphocytes (PBL) have shown incredible results in B cell malignancies, with tolerable on-target toxicities. For acute myeloid leukemia (AML), however, a suitable target antigen that is expressed on leukemic blasts and preferably also leukemic stem cells, and not on indispensable normal hematopoietic cells, has yet to be discovered. Target antigens in current (pre)clinical studies show important on-target off-leukemia toxicity, with myelotoxicity and impaired hematopoiesis being the main concern.
Therefore, we are focusing our research on T cell receptor (TCR)-based immunotherapy. Our strategy to generate large numbers of mature tumor antigen-specific T cells with a single TCR and naive-like characteristics, is based on the OP9-DL1 in vitro coculture system to induce T cell differentiation from $CD34^+$ hematopoietic stem cells (HSC). We have already shown the feasibility of this method, starting from postnatal thymus and cord blood HSC, and have now optimized our protocol for clinically relevant samples, such as mobilized peripheral blood from adult healthy donors and from patients in remission after chemotherapy, and leukapheresis and peripheral blood samples from AML patients at diagnosis with a hyperleucocytic CD34 negative AML.
$CD34^+$ HSC were isolated from in total 9 healthy donors, 11 patients in remission after chemotherapy, and 7 AML patients at diagnosis. These HSC were cultured on the OP9-DL1 cell line in the presence of cytokines, until T cell commitment. Subsequently, the cells were transduced with a tumor antigen-specific TCR and again cocultured until $CD4^+$ $CD8^+$ double positive (DP) cells were abundantly present. At that point, the agonist peptide, which is recognized by the TCR, was added to induce maturation of HLA-$A2^+$ samples, while HLA-$A2^-$ samples were cocultured with a cell line presenting the peptide. Finally, the cells were polyclonally expanded on irradiated feeder cells. Part of the cells from the initial patient and healthy donor samples were frozen and later thawed to evaluate the effect of freezing and thawing of HSC on T cell maturation.
Using the above protocol, we were able to generate tumor antigen-specific T cells from adult patient and healthy donor stem cells. However, for most samples, maturation kinetics were slower compared to cord blood. Also, multiple rounds of agonist peptide stimulation were necessary to achieve selection

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and maturation of HSC from adult sources. This is in contrast to generation of mature T cells from cord blood HSC, for which we only need one round of agonist peptide stimulation. Furthermore, we showed that cryopreservation slows down expansion of T cell precursors in in vitro cultures. At this moment, we are evaluating the in vitro and in vivo functionality of our in vitro generated T cells. These experiments are a necessary step before we can bring this novel patient-tailored T cell immunotherapy to the clinic.

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Automated ex vivo expansion of low numbers of tumor-reactive T cells on the CliniMACS Prodigy®

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Adoptive cell transfer (ACT) of tumor-infiltrating lymphocytes (TILs) have shown remarkable results in patients with metastatic melanoma. However, usually only a small fraction within the TIL population reacts against the tumor. Therefore, the pre-enrichment of tumor-specific T cells and subsequent ex vivo expansion may improve the efficiency of ACT therapies. In addition, tumor-reactive T lymphocytes circulating in the blood (TRLs) have been found in low frequencies, which represents a challenge for their isolation, but also an advantage over TIL therapy since blood is a more reliable and accessible source than tumor excisions. Another impediment to the widespread application of ACT is the conventional rapid expansion protocol (REP) that constitutes a laborious and extensive process with frequent culture manipulations, and thus requires specialized personnel and equipment.
Our aim is to develop a fully automated large scale ex vivo T cell isolation and expansion procedure in the CliniMACS Prodigy in order to simplify the manufacturing of tumor-reactive T cells for ACT. The CliniMACS Prodigy instrument is a controlled system that integrates a series of cell processes, from magnetic cell separation and cell culture to final product formulation, under GMP conditions in a closed system. This process focuses on the optimization of the REP procedure on the CliniMACS Prodigy for TILs and TRLs. As a proof of concept, we used both cryopreserved outgrown TILs and magnetically isolated virus-specific T cells from healthy donor leukapheresis via CD137 upregulation upon in vitro antigen stimulation. The first results show expansions up to 4,000 fold, both for TILs and CD137-expressing T cells. From low cell numbers ($1x10^6$ cells) and after 14 days of cell culture in TexMACS GMP medium in the presence of high amounts of IL-2 and irradiated feeder cells, we were able to obtain around $3\text{-}4x10^9$ cells. We also compared two different stimulation reagents, MACS GMP CD3 pure antibody (OKT3 antibody) and MACS GMP T cell TransAct (a soluble polymeric nanomatrix conjugated to humanized CD3 and CD28 agonist), which resulted in comparable expansion rates. Furthermore, small scale experiments showed no differences between TexMACS and the conventional TIL culture medium (50% RPMI/50% AIM-V medium). The phenotype and reactivity of the expanded T cells were also assessed by flow cytometry.
In summary, these data provide proof of concept for the expansion of low numbers of TILs and virus-specific T cells from peripheral blood in a closed, automated manner in the CliniMACS Prodigy. In the future, this expansion process will be combined with a tumor-reactive T cell enrichment step (e.g. via CD137-conjugated magnetic beads) to achieve the desired efficiency, simplicity and automated production of ACT therapies against cancer.

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Nanobody based dual specific CARs

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Recent clinical trials have shown that adoptive chimeric antigen receptor (CAR) T cell therapy is a very potent and possibly curative option in the treatment of B cell leukemias and lymphomas. However, targeting a single antigen may not be sufficient, and relapse due to the emergence of antigen negative leukemic cells may occur. A potential strategy to counter the outgrowth of antigen escape variants is to broaden the specificity of the CAR by incorporation of multiple antigen recognition domains in tandem. As a proof of concept, we have generated a bispecific CAR in which the single chain variable fragment (scFv) were replaced by a tandem of two single-antibody domains or nanobodies (nanoCAR). High membrane nanoCAR expression levels are observed in retrovirally transduced T cells. NanoCARs specific for CD20 and HER2 induce T cell activation, cytokine production and tumor lysis upon incubation with transgenic Jurkat cells expressing either antigen or both antigens simultaneously. The use of nanobody technology allows for the production of compact CARs with dual specificity and predefined affinity.

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Molecular mechanisms regulating TCR affinity-improved T cells against the NY-ESO-1 tumor antigen

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Background and Rationales: T cell receptor (TCR) affinity for its cognate antigen (pMHC) is a key parameter controlling T cell potency, with stronger TCR-pMHC interactions conferring superior T cell activation and function than weaker ones. Redirecting CD8 T cell immunity with tumor-specific affinity-increased TCRs in adoptive T cell therapy results in improved anti-cancer efficacy, but also in harmful autoimmune events. Despite numerous functional-based studies, the characteristics of TCR signaling (i.e. intensity, duration) and the regulatory mechanisms underlying optimal therapeutic T cell responses remain poorly understood. Using a panel of human SUP-T1 and primary CD8 T cells engineered with incremental affinity TCRs against the NY-ESO-1 tumor antigen, we aimed at dissecting the impact of TCR-pMHC affinity on the proximal (CD3ζ) and distal (ERK1/2) TCR signaling nodes as well as to evaluate the regulatory roles of SHP phosphatases on those early TCR signaling events.
Results: Upon antigen-specific TCR triggering, T cells with optimal-affinity TCRs yielded intense and sustained CD3ζ activation levels resulting in maximal ERK1/2 activation intensity and duration, associated to increased function. In contrast, T cells with very high affinity TCRs displayed rapid and strong signal initiation but only transiently, leading to poor MAPK activation and low proliferation potential. We found that these hyporesponsive T cells present readily, under resting conditions, significantly reduced levels of surface TCR/CD3ε, CD8β and CD28 expression and of CD3ζ phosphorylation. SHP phosphatases were also involved in calibrating optimal TCR signaling, but exhibited distinct roles in the TCR signaling transduction cascade. SHP-1 phosphatase activity controlled TCR signaling initiation and subsequent amplification by inhibiting CD3ζ and ERK1/2 phosphorylation, in a TCR affinity-dependent manner. SHP-2 phosphatase had a more positive impact on TCR signaling by sustaining downstream ERK1/2 phosphorylation without directly affecting TCR/CD3ζ activation.
Conclusions: Our findings indicate that optimal TCR signaling can be finely tuned by TCR affinity-dependent SHP-1 and SHP-2 activity. Importantly, TCR-ligand affinity modulates the expression and phosphorylation levels of the TCR/CD3 complex readily under steady-state conditions. Thus, engineered CD8 T cells of very high affinity but not of optimal TCR affinity present a TCR affinity-mediated hyporesponsive state. Since the gain in TCR affinity was mainly related to mutations in the

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CDR2 loops, mostly interacting with the HLA-A2 backbone, we are currently investigating whether, in the very high affinity T cells, weak but chronic TCR-HLA-A2 binding interactions, even in the absence of cognate peptide may result in this hyporesponsive state. Together, our observations should help to define the best TCR signaling-mediated characteristics and promote the use of therapeutically affinity-engineered CD8 T cells.

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Balancing CD28 costimulation and CD3ζ activation in CD19 CAR T cells enhances therapeutic potency by delaying T cell differentiation and exhaustion

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Immunotherapy with second generation chimeric antigen receptor (CAR) T cells has achieved great clinical success against hematological malignancies with induction of durable remissions. Despite these promising results, suboptimal responses with relapses occur. We hypothesized that excessive signaling strength due to combined CD3ζ activation and CD28 costimulation in 1928ζ CARs might lead to early T-cell differentiation and exhaustion, thus diminishing antitumor activity. We therefore analyzed the contribution of individual signaling domains to overall CAR function, titrating CAR signaling to limit overstimulation of CAR T cells but without compromising anti-tumor activity.
We introduced defined mutations in the CD28/CD3ζ signaling modalities of 1928ζ CARs, disabling full activation of downstream signaling pathways via the mutated signaling domains. T cells were effectively transduced using SFG retroviral vectors achieving comparable transduction rates for all the different constructs. Functional in vitro and in vivo analyses demonstrated graded differences in the therapeutic potency of 1928ζ mutants depending on the mutation. Disruption of some signaling domains in 1928ζ CAR led to diminished effector function as reflected by rapid tumor growth and reduced survival of treated mice. In vitro analyses confirmed diminished cytolytic potential and revealed reduced Th1 cytokine and Granzyme B secretion of these 1928ζ mutants. However, we also identified 1928ζ mutant CARs that consistently emerged to be superior to 1928ζ WT CARs, achieving rapid and durable tumor eradication even at low T cell infusion doses. Improved T cell function was associated with delayed T-cell differentiation, increased proliferative potential and reduced T cell exhaustion in these 1928ζ mutants. In vivo studies revealed enhanced CAR T cell accumulation at the tumor sites associated with a significant increase in the percentage of central memory cells and a decreased proportion of terminally differentiated effector cells.
In conclusion, we show that distinct mutations within the CD28/CD3ζ domain differentially affect functional properties of 1928ζ CAR T cells. We identified novel 1928ζ CAR T cells that precisely fine-tune the strength of CAR signaling to levels that preserve critical functional properties and augment functional persistence of CAR T cells. These superior CAR designs lead to significantly enhanced tumor rejection in mice and are thus great candidates for future clinical trials.

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Selecting CD8-specific DARPins for T lymphocyte-directed gene delivery

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Delivering genes selectively to the therapeutically relevant cell type is among the prime goals of vector development in the field of gene therapy. Receptor-targeted lentiviral vectors (RT-LVs) were shown to be promising tools for directed gene delivery *in vitro* and in *in vivo* systems. Among the most advanced vectors described to date is an LV displaying a CD8-specific single-chain variable fragment (scFv). This vector enables highly specific gene transfer to human $CD8^+$ cytotoxic T cells in a xenograft mouse model (Zhou et al., Blood 2012). While these results are encouraging, higher titers to increase transduction rates as well as targeting domains enabling the *in vivo* genetic modification of $CD8^+$ T cells in syngeneic mouse models and lager animals like non-human primates (NHP) would be desirable.
Designed ankyrin repeat proteins (DARPins) have become an attractive alternative to scFv domains. Derived from combinatorial DARPin libraries, high affinity binders for almost any target of choice can be selected by ribosome display. Importantly, DARPins can act as targeting ligands for RT-LVs, mediating highly specific gene transfer vectors with improved titers.
Here we show the complete process of DARPin selection starting from ribosomal display, over the screening of candidates for target cell binding up to the identification of DARPins working best for CD8-targeted gene delivery with LVs. In particular, we selected DARPins specifically binding to both human and primate CD8 as well as DARPins recognizing murine CD8. For the ribosome display selection procedure we produced human and murine CD8 as metabolically biotinylated Fc-fusion proteins in HEK-293T cells. On-target selection on immobilized CD8 was followed by off-rate selection in presence of soluble CD8 to enrich high-affinity binders. Distinct selected DARPins were expressed in bacteria for CD8 binding analysis on primary human, primate and murine $CD8^+$ cells. This way, promising candidates binding to both human and primate CD8 as well as to murine CD8 were identified. All candidates could be efficiently displayed on the surface of lentiviral particles, resulting in high titers, high transduction efficiency and exquisite specificity for $CD8^+$ target cells. Importantly, the CD8-DARPin-LVs were as specific for their targets as their scFv-based counterparts, but more efficient in mediating gene transfer into primary $CD8^+$ cells. These novel RT-LVs will enable targeting of cytotoxic T cells *in vivo* in xenograft mouse models and NHP using the same targeting domain as well as in syngeneic mouse models in future.

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The effect of anti-CTLA-4 blockade on the expansion of tumor-infiltrating lymphocytes for adoptive cell therapy in metastatic ovarian cancer

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Introduction: The use of *in vitro* expanded tumor-infiltrating lymphocytes (TILs) in adoptive cell therapy (ACT) has been shown to induce complete and durable tumor regression in patients with advanced melanoma. Efforts are currently underway to expand this treatment modality to other cancer types.
The accumulation of TILs in ovarian cancer is prognostic for increased survival while increases in immunosuppressive regulatory T cells are associated with poor outcomes. Within the tumor microenvironment, regulatory cells and expression of co-inhibitory immune checkpoint molecules can lead to the inactivation of TILs. Recently, it has been established that anti-CTLA-4 therapies can mediate antibody-dependent cell-mediated cytotoxicity and that CTLA-4 is expressed on the surface of tumor-infiltrating regulatory T cells. Thus, approaches that directly manipulate co-stimulatory pathways within the initial tumor fragment cultures might improve the expansion of tumor-resident TILs enriched

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for tumor specificity.
Here, we wanted to test the effect of anti-CTLA-4 blockade on the expansion of TILs by manipulating the initial tumor fragment cultures from patients with metastatic ovarian cancer.
Methods: In this study, we hypothesized that the blockade of the CTLA-4 co-stimulatory pathway in ovarian tumor fragments enhances CD8+ T-cell output and TIL tumor reactivity. Ipilimumab, a CTLA-4-targeting antibody, was added during the initiation of the TIL expansion process and/or during the rapid expansion phase and the phenotype and functionality were analyzed by flow cytometry.
Results: Preliminary data show that blockade of CTLA-4 added during the initiation of TIL cultures increased the rate of CD8+ TIL expansion, which is preserved during the rapid expansion phase. Additionally, the tumor reactivity of the TILs expanded from fragments where ipilimumab was added to the culture showed increased tumor reactivity in both young TILs and rapidly-expanded TILs compared to TILs not cultered with ipilimumab.
Conclusion: Our data suggest, that targeting CTLA-4 within the initial tumor fragment favor the expansion of CD8+ TILs and that this manipulation could potentially improve TIL products with respect to phenotype and functionality.

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In vivo generation of CD4+ and CD8+ CAR-T cells by receptor-targeted lentiviral vectors

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T cells modified with CD19-specific chimeric antigen receptors (CARs) result in significant clinical benefit for leukemia patients. However, production of CAR T cells requires extensive and time-consuming procedures of cell isolation, sorting, transduction and *in vitro* expansion of T cells. Receptor-targeted lentiviral vectors (LV), which transfer genes selectively into particular types of lymphocytes may enable direct *in vivo* CAR gene delivery, thus simplifying the process. Here, we focus on the *in vivo* generation of CD4+ as well as CD8+ CAR T cells using CD4- (CD4-LV) and CD8-targeted (CD8-LV) lentiviral vectors, since there is increasing evidence that besides cytotoxic CD8+ T cells, also cytokine secreting CD4+ T cells are needed for successful CAR T cell therapy.
Both, CD4-LV and CD8-LV transferred reporter genes specifically into lymphoid tissues and selectively into CD4+ and CD8+ T cells, respectively, when administered intravenously into NSG mice engrafted with human lymphocytes. Cultivated huPBMC were transduced with the CD19-CAR gene by either CD4- or CD8-LV or the combination of both resulting in the generation of CAR T cells. Both types of CAR T cells proliferated efficiently upon antigen exposure. CD8 CAR T cells specifically killed target cells, while analyzing the killing activity and cytokine secretion of CD4 CAR T cells is still ongoing. For the *in vivo* generation of CAR-T cells, NSG mice were i.p. injected with human PBMC (containing CD19+ B cells) followed by administration of CD4- or CD8-LV delivering the CD19-CAR. One week after vector injection mice were sacrificed and peritoneal cells as well as spleen and blood cells were analyzed by flow cytometry. Interestingly, vector treated mice contained substantially more CD4+ cells following CD4-LV treatment and substantially more CD8+ cells after CD8-LV injection than control animals. Notably, about 40-60% of the CD4+ cells and 20-50% of the CD8+ cells, isolated from the peritoneum were CAR-positive, while no CAR cells were detected in the CD4- and CD8- cell fraction respectively. Moreover, control mice contained about 0.2-2.5% CD19+ cells floating in the peritoneal cavity, while no CD19+ cells could be detected in CD4-LV or CD8-LV treated mice, indicating that both types of *in vivo* generated CAR-T cells were functionally active.
Our results show that distinct subtypes of CAR T cells can be generated *in vivo* using receptor-targeted LVs. Future activities focus on investigating the anti-tumor potential of *in vivo* generated CAR T cells and transferring the system to CD34+ humanized mice, better resembling the clinical relevant situation.

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The first two authors contributed equally to this work.

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Intracerebral immunomodulation using genetically engineered mesenchymal stem cells induces long-term survival and immunity in glioblastoma

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Glioblastoma (GBM) represent one of the most aggressive brain tumors. Standard therapy consists of surgical removal followed by radiation and chemotherapy. Due to its invasive growth all tumors relapse, which correlates with a dismal prognosis and an average survival period of 12 to 14 month after diagnosis. Mesenchymal stem cells (MSCs) show an inherent brain tumor cell tropism that can be exploited for targeted delivery of therapeutic genes to invasive glioma. We assessed whether a motile MSC-based local immunomodulation is able to overcome the immunosuppressive GBM microenvironment and to induce an antitumor immune response.
apceth-301 is a cell-based immunotherapy consisting of allogeneic MSCs which are genetically modified to co-express high levels of IL-12 and IL-7. In vitro characterization demonstrated that apceth-301 increased T-cell activation, as measured by increased secretion of interferon gamma and tumor necrosis factor alpha. Furthermore, the MSCs promoted NK cell mediated killing of GBM cell lines in co-culture assays. MRI-based cell tracking and histological analysis confirmed a rapid and targeted tumor tropism towards glioblastoma xenografts. Therefore, therapeutic efficacy of apceth-301 was assessed in an immunocompetent orthotopic GL261/C57Bl6 glioblastoma model. Intratumoral administration of murine MSC-IL-12/7 at day 5 or day 10 induced a significant tumor growth inhibition and displayed more intratumoral T2-hypointensities on MRI, suspicious for tumor necrosis. All control animals died by day 28, while more than 50% of treated mice survived long-term (>100d). MRI in long-term survivors after a single treatment with murine MSC-IL-12/7 did not reveal any tumor mass and demonstrated a clear tumor regression when compared with initial MRIs. Re-challenging survivors with another intracerebral injection of GL261 confirmed long-lasting tumor immunity. Whereas naive control animals died quickly as expected, re-challenged survivors did not develop any signs of tumor growth on MRI and displayed long-term survival. Immunomodulatory effects were assessed in a separate experiment by immunohistology and flow-cytometry at different time points to comprehensively profile immune activation of tumor-infiltrating lymphocytes and peripheral blood lymphocytes. Local treatment with MSC-IL12/7 was well tolerated and led to a significant switch of CD4+/CD8+ T-cell ratios compared to control animals with an intricate predominantly CD8+ T-cell mediated anti-tumor response.
Local MSC-based delivery of immunomodulatory cytokines is well tolerated and able to efficiently alter the immunosuppressive microenvironment in glioblastoma resulting in tumor immunity. The significant and long lasting therapeutic effects warrant a rapid clinical translation of this concept and have led to planning of a phase I/II study using apceth-301.

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Affinity maturation and validation of a COL6A3-002-specific T cell receptor for improving performance in adoptive cell therapy

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Adoptive cell therapy (ACT) with T cell receptor (TCR)-engineered T cells has emerged as a promising therapeutic modality. In contrast to chimeric antigen receptor (CAR)-based therapy, the utilization of a TCR allows targeting of human leucocyte antigen (HLA)-bound peptides derived from virtually all proteins of the tumor cell regardless of their extracellular or intracellular location. Immatics is developing TCR-engineered ACT against tumor-associated peptide-HLA targets, which have been identified and validated by its proprietary target discovery engine XPRESIDENT®. Immatics has further established a portfolio of technologies to discover and engineer TCRs originating from the natural repertoire of human donors for optimizing performance of TCR-engineered therapeutic approaches.
Here we present the affinity maturation and validation of a TCR directed against the tumor-specific peptide COL6A3-002 presented on HLA-A*02. The parental TCR selected for maturation has previously been identified and validated for its highly specific recognition of COL6A3-002 while binding affinity towards COL6A3-002 was rather low with a measured equilibrium dissociation constant of 62 µM. After converting the TCR into a single chain format comprising the variable domains connected by a peptide linker (scTv), yeast scTv libraries were generated for stability and affinity maturation by introducing mutations in scTv framework and CDR sequences, respectively. Selection of improved scTv candidates was performed via MACS and multicolor FACS employing increased selection stringency. Nine maturated scTv candidates derived from 2 CDR libraries showed improved binding properties resulting in up to 300-fold increased affinity towards COL6A3-002:HLA-A*02. While several maturated scTv variants showed increased binding to highly similar off-target peptides whose presentation on healthy tissue was confirmed by XPRESIDENT®, two maturated scTv variants remained highly specific for COL6A3-002. Reintroducing the TCRs into CD8+ T cells confirmed an improved COL6A3-002 recognition of the maturated TCR variants when compared to the parental TCR, which was quantified by IFN-γ release. Analysis of the maturated TCR variants upon expression in CD8+ T cells furthermore highlighted the superior specificity profile of two mutant TCR variants showing no cross-reactivity towards any of the highly similar off-target peptides. Our data demonstrate the successful application of Immatics´ TCR engineering platform to generate affinity maturated and highly specific TCRs against selected tumor-associated peptide-HLA targets for improved performance in ACT.

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CD4 T-cell based immunotherapy of cancer

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Whereas cellular anti-tumour immune responses have typically been attributed to CD8 T cells, CD4 T cells play a critical role in tumour elimination and the priming and maintenance of CD8 T-cell responses. CD4 T cells can modulate CD8 T-cell migration and produce cytokines that directly impact exhausted CD8 T cells. Furthermore, CD4 T cells activate innate cells such as macrophages and NK cells to contribute to anti-tumour responses.
The use of MHC class II-restricted CD4 T cells for adoptive immunotherapy has been limited due to i) a lack of well-characterized shared tumour antigens presented by MHC class II ii) the majority of tumour cells being class II negative and therefore not directly presenting antigen to CD4 T cells.
We have isolated CD4 T cells reactive against tumour antigens from patients who experienced clinical benefit from treatment with cancer vaccines targeting universal tumor antigens and frequent neoantigens. Strong T-cell responses correlated with enhanced survival and tumour regression in such late stage cancer patients. These HLA class II-restricted T-cell clones recognised target cells loaded with long peptides or protein and some CD4 T-cell clones could also directly recognize tumour recognition.
When TCRs were isolated and expressed in donor T cells we found that they were functional in both

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CD4 and, surprisingly, in CD8 T cells, producing TNF-α and IFN-γ with the capacity of target cell killing. Our preliminary in vivo data indicate that TCR recognizing the universal antigen, hTERT could control MHC-II tumour growth.
We believe that combining HLA class I- and class II-restricted TCRs for T-cell redirection may provide a more potent therapeutic effect in adoptive T cell therapy. HLA class II-restricted TCRs may additionally have direct therapeutic value both in haematopoietic malignancies and in melanoma where tumour cells frequently express HLA class II. Importantly, CD4 T cells and HLA class II TCRs therefore have the potential to orchestrate broad and long-lasting immune responses that enable cancer control.

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Substantially enhanced CAR-gene transfer rates by CD4- and CD8-targeted lentiviral vectors while retaining selectivity

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Chimeric Antigen Receptor decorated T cells are an ingenious personalized cell therapy with proven clinical benefit in hematological malignancies. However, this novel treatment still needs to overcome various hurdles including a complicated manufacturing process. Lentiviral vectors (LVs) are one of the most favorable vectors for CAR gene delivery. Receptor-targeted LVs, such as CD4-LV and CD8-LV, delivering genes selectively to T cell subtypes may facilitate and improve CAR T cell generation but so far result in lower gene delivery rates than conventional LVs pseudotyped with the vesicular stomatitis glycoprotein (VSV-LV). Here, we study the influence of the transduction enhancer Vectofusin-1 on gene delivery of a second generation CD19-CAR and ΔLNGFR as a reporter gene to human T cells with CD4- and CD8-targeted LVs.
All particles were produced at high concentration ($4x10^{11}$ - $2x10^{12}$ particles/ml) and each batch was titrated on cell lines. Afterwards, vectors were used to transduce activated human primary peripheral blood mononuclear cells (PBMC) in the presence or absence of the transduction enhancer Vectofusin-1.
Transduction was performed with PBMC of 6-7 healthy donors and four (CD4-LV and CD8-LV) or two (VSV-LV) vector batches. Vectofusin-1 enhanced the mean transduction rate from 49% to 74% for CD4-LV and from 46% to 78% for CD8-LV. In some samples transduction rates approached 100%. Interestingly, transduction rates of VSV-LV were lower in presence of Vectofusin-1 (20-40%) than in absence (55-80%). To determine the functionality of the CAR T cells generated in the presence of the transduction enhancer, killing assays and quantification of released cytokines are currently ongoing. Preliminary data show that Vectofusin-1 does not impair the killing competence of the generated CAR T cells.
Interestingly, although target cell specificity was retained when cultivating the transduced T cells for almost two weeks, a transient off-target signal for ΔLNGFR in the presence of Vectofusin-1 was observed at earlier time points. To assess if this signal was caused by protein transfer, we incubated activated PBMC for 1 hour at 4˚C with CD4-LV or CD8-LV having packaged the CD19-CAR and ΔLNGFR genes. Notably, ΔLNGFR was readily detectable on target-receptor positive cells as well as off-target cells by flow cytometry even in absence of Vectofusin-1. Increased washing steps during the transduction procedure resulted in decreased off-target positivity. ELISA assays on vector particles confirmed the presence of about 144 molecules of ΔLNGFR per vector particle.
The data demonstrate that transduction enhancers can substantially raise gene delivery rates with CD4-LV and CD8-LV thus being in the range or even above that achievable with VSV-LV. This increase in gene delivery comes without affecting target cell selectivity; although a transient protein delivery to off-target cells can occur.

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First two authors contributed equally to this work.

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Best chimeric antigen receptor (CAR) expressing vehicle: peripheral versus tumour infiltrating lymphocytes

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Great successes have been reported in haematological malignancies using CD19 chimeric antigen receptor (CAR) T cells leading to the FDA approval, in August 2017, of this therapy for patients with chemorefractory and recurrent acute lymphoblastic leukaemia (ALL). The successes using CAR T cells in solid tumours have not been as impressive as with the haematological malignancies. Adoptive cell therapy utilising tumour infiltrating lymphocytes (TILs) in the treatment of mainly melanoma patients showed great promise. The limited applicability of TILs in other solid tumours was associated with the lack of pre-conditioning and the failure to expand cells. Whereas, the limited success of peripheral T cells endowed with CARs in solid tumours could be attributed to the lack of tumour homing receptors as well as natural tumour reactivity as well as the immunosuppressive microenvironment. Therefore, we hypothesise that CAR expressing TILs will have a higher homing and infiltration potential as well as maintain natural tumour reactivity which will act in combination with CAR targeting, leading to complete tumour eradication. In order to test this hypothesis TILs and peripheral T cells from the same patients will be transduced with CARs and tested against autologous tumour in *in vitro* and *in vivo* settings. Initially, different solid tumour samples, such as colorectal and ovarian cancer, were digested using the gentleMACS™ dissociator and the cells were used to isolate and expand TILs. TILs and peripheral T cells from the same patient were transduced with carcinoembryonic antigen (CEA) specific CARs to determine any differences in the functionality against CEA expressing cell lines using cytokine detection assays. The results show a positive correlation between the weight of the tumour sample and the number of cells isolated. The population of T cells in the periphery possess a lower frequency of Tregs and lower exhaustion and activation surface marker expression when compared to the TILs. TILs have been successfully generated from colorectal (65%) and ovarian cancer (77.8%) samples and responsiveness to autologous tumour has been observed in 6/15 (40%) and 5/6 (83.3%) of the colorectal and ovarian cancer samples, respectively. Direct comparison of CAR-T and CAR-TIL has been successfully achieved and the results indicate differences in cytokine production, however, the differences can be attributed to donor variation. Interestingly, CAR-TILs also possess less non-specific reactivity to cell lines compared to CAR T cells. Current analysis involves further dissection of the differences between the two populations including the assessment of their infiltration and homing capacity to determine the CAR vehicle for solid tumours.

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Adoptive immunotherapy using DNA-demethylated Th-cells as antigen presenting cells induces tumor regression in patients with relapsed glioblastoma

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Cancer/testis (CT) antigens are expressed in cancer cells of different histological origin due to demethylation of the corresponding gene promoters. These antigens could be used as targets for anti-tumor immunotherapy, however, an immunization strategy targeting a broad spectrum of CT antigens is required to overcome the heterogeneity of CT-antigen expression among and within tumors. We have used polyclonally activated CD4+ T-helper (T_H) lymphocytes as antigen-presenting cells. A broad spectrum of CT antigens in these cells was expressed after treatment with the DNA-demethylating agent 5-aza-2´-deoxycytidine. Employment of such T_H cells for in vitro immunization of autologous lymphocytes resulted in the generation of immune lymphocytes enriched in CD8+ cytotoxic T lymphocytes (CTLs) and NK cells. T cell receptor clonotype analysis showed clonal expansion of T lymphocytes, indicating a successful immunization process. Isolated CTLs were able to kill a broad spectrum of human tumor cell lines in an HLA class I-restricted manner. A significant part of the CTLs expressed CD62L and CD27, indicating a central memory type CTL response. ELISPOT analysis of antigen specificity of the isolated CTLs demonstrated recognition of two known CT antigens: NY-ESO-1 and MAGE-A10. The efficiency of this technology was tested in a phase 1 clinical trial of patients with relapsed glioblastoma, without lymphodepletion. The injected lymphocytes homed to the tumor site. Disease control was achieved in six out of 25 patients included in the study. In three of these patients, tumor regression was ongoing for 14, 22 and 27 months, respectively, with one complete response. We conclude that epigenetically modified T_H cells can be used to generate autologous tumor-reacting lymphocytes, which may provide a new, general strategy for cancer therapy. This approach can be combined with check-point inhibitors, as well as with systemic treatment using DNA-demethylating agents. Provided that the induced immune response is persistent and has no long-term adverse effects in healthy persons, the present approach may also open new possibilities for cancer prevention.
Reference: Kirkin A.F. et al. *Nature Communications* (in press)

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Selective bispecific antibodies enable synthetic agonistic receptor-transduced T cells for tumor therapy

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Background: Adoptive T cell therapy (ACT) using chimeric antigen receptor-transduced T cells is an effective strategy to treat hematological malignancies. Broader application of ACT is hampered by limited efficacy in solid tumors and by side effects. We herein describe a novel MHC-unrestricted modular platform for effective and safer ACT with potential for clinical development. The platform combines autologous T cells, transduced with a targetable synthetic agonistic receptor (SAR), with crosslinking bispecific antibodies that specifically recruit and activate T cells to the tumor.
Methods: Bispecific antibodies (BiAb) of different formats were generated by recombinant expression cloning. T cells were retrovirally transduced with synthetic agonistic receptors. T cell activation, proliferation, differentiation and T cell induced-lysis were characterized in three murine and three human tumor models *in vitro* and *in vivo*.
Results: Murine T cells transduced with two different SARs composed of an extracellular domain (EGFRv3 or Cripto-1) fused to the intracellular domains of CD28 and CD3z could be specifically recruited towards murine tumor cells expressing EpCAM by anti-EGFRv3 (or anti-Cripto-1) x anti-EpCAM BiAb. BiAb induced selective antigen-dependent activation, proliferation of SAR-T cells and redirected tumor cell lysis by the T cells. Selectivity was dependent on the monovalency of the

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antibody for the antigen transduced into the T cell. We dissected the mode of action, identifying FAS ligand as the major mediator of killing utilized by the T cells. SAR-T cells administered in combination with BiAb mediated anti-tumoral activity *in vivo*. Similarly, human T cells transduced with the synthetic agonistic receptor EGFRv3 - CD28 - CD3z could be specifically redirected towards mesothelin-expressing human pancreatic cancer cells through the respective BiAb. *In vivo,* the combined administration of SAR-T cells and BiAb mediated anti-tumoral activity in a human pancreatic cancer cell xenograft model.
Conclusions: We describe a novel ACT platform with anti-tumor activity in murine and human tumor models with a distinct mode of action compared to other ACT modalities. The dependence on BiAb triggering may enhance both efficacy and the safety profile.

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Arming T cells with C-X-C-motive receptor 6 enables adoptive T cell therapy of cancer
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Background: Adoptive T cell therapy (ACT) using chimeric antigen receptors has been established as a powerful treatment for hematologic malignancies. In solid tumors, however, efficacy remains more limited. One of the major hurdles to ACT efficacy is the access of the transferred T cells to tumor tissue. We hypothesized that this limitation might be overcome by *ex vivo* transducing a chemokine receptor into T cells before ACT.
Methods: Chemokine expression profiles were analysed in murine and human pancreatic cancer cell lines. Murine and human T cells were engineered using retroviral transduction. Migration was analyzed in vitro in transwell assays and in vivo using flow cytometry and 2-photon microscopy. T cell efficacy was tested in real-time-impedance measurement, ELISA and in vivo in syngeneic and xenograft tumor models.
Results: We identified CXCL16 as a chemokine highly expressed by both human and murine pancreatic cancer cells while its receptor, C-X-C-receptor 6 (CXCR6), is absent from cytotoxic T cells. Introducing CXCR6 into primary murine and human T cells increased T cell migration towards CXCL16 gradients both in vitro and in vivo. T cells armed with CXCR6 exhibited enhanced tumor cell recognition and lysis due to the adhesive effect of CXCR6 - CXCL16 interactions. In vivo, T cells bearing either a T cell receptor (TCR), a murine chimeric antigen receptor (CAR) targeting EpCAM or a human CAR targeting mesothelin only mediated sustained anti-tumoral activity when combined with the forced expression of CXCR6 on the T-cells. Enhanced anti-tumoral activity was accompanied by increased T cell influx to the tumor tissue as demonstrated by 2-photon microscopy, as proposed main mode of action of CXCR6 on cytotoxic T cells.
Conclusions: CXCR6 enables T cells efficacy against a panel of tumor models of murine and human origin. CXCR6 enhances efficacy of T cells armed with either a TCR or a CAR specific for the cancer cell. This provides a strong rationale for further translation of the approach.

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Murine-human hybrid tyrosinase reactive T-cell receptor increases antigen reactivity and cytokine production

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Adoptive transfer of engineered T cells is a promising approach for cancer treatment. One method is to modify T cells to express a tumor antigen-specific T cell receptor (TCR). While this approach is advantageous for limiting the T cell target to tumor-associated antigens, not all patients that have received TCR-engineered T cells show sufficient tumor regression. This is due in part to engineered T cells expressing a low level of the tumor antigen-specific TCR and having reduced functional capacity. Thus, there is a great interest in increasing specificity and reactivity of these engineered tumor-specific TCRs.
Previous works by others demonstrated that a murine-human hybrid TCR, which is a fusion between the human TCR variable region with the mouse TCR constant region, provokes a highly elevated antigen-specific T cell response when compared to wild-type human TCRs. This is because the mouse TCR constant region pairs more efficiently with the human CD3 complex than its human counterpart. As a result, surface expression of the engineered TCR is higher and induces better signaling events than the wild-type TCR.
The goal of this study is to explore the potential of murine-human hybrid TCR for clinical applications. For this purpose, we generated a new murine-human hybrid receptor that recognizes human tyrosinase, an antigen that is expressed in melanoma lesions. We transduced primary T cells with the tyrosinase-specific hybrid TCR using a retroviral plasmid. The hybrid TCR expressing T cells secreted higher amounts of cytokines, such as IL-4, IL-5, IL-13, IL-22, IL-10 and IFN-γ, than the wild-type TCR transduced T cells upon antigen stimulation. Compared to wild-type TCR transduced T cells, murine-human hybrid TCR transduced T cells expressed higher levels of transduced TCR and required a lower concentration of peptide stimulation to promote cytokine production. Together, these data showed that our tyrosinase-specific murine-human hybrid TCR has higher antigen reactivity and cytokine production than wild-type human TCRs. We are currently investigating *in vivo* anti-tumor efficacy of the hybrid receptor-expressing T cells.

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PD-L1-specific immunocytokines augment functionality and antitumor activity of CAR-engineered NK cells

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Natural killer cells play an important role in cancer immunosurveillance, with their cytotoxicity triggered rapidly upon stimulation through germline-encoded cell surface receptors. In addition, NK cells modulate adaptive antitumor immunity by maintaining the quality of dendritic cells and improving presentation of tumor antigens. Genetic engineering of NK cells with chimeric antigen receptors (CARs) can enhance specific recognition and selective elimination of tumor cells. Nevertheless, expression of programmed death receptor-ligand 1 (PD-L1) by tumor cells may dampen the CAR NK cells´ direct and indirect antitumor activity.
To overcome immunosuppressive effects, we aim to develop advanced CAR NK cells, which secrete PD-L1-specific antibody-cytokine fusions that carry IL-12 or IL-15. These immunocytokines can be retained in the tumor microenvironment by binding to PD-L1 on the tumor cell surface, and may simultaneously block the PD-1/PD-L1 immune checkpoint and activate innate and adaptive bystander immune cells. For initial testing of their functionality, antibody-cytokine fusion proteins were expressed

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in HEK293 cells and purified from culture supernatants. Binding of the recombinant proteins to PD-L1 and activity of their IL-12 and IL-15 domains were verified by flow cytometry and in bioactivity assays. The immunocytokines enhanced T-cell activation in mixed lymphocyte reactions and increased cytotoxicity of NK cells against tumor cells. Subsequently, PD-L1-specific antibody-cytokine fusions were introduced into ErbB2 (HER2)-specific CAR NK-92 cells by lentiviral transduction. The ectopically expressed immunocytokines activated the respective cytokine signaling pathways in the producer cells, in the case of anti-PD-L1-IL-15 resulting in cell growth and activity becoming independent from exogenous IL-2. Furthermore, in transwell assays stimulatory effects on different types of co-cultured immune cells were observed. Our results show that PD-L1-specific IL-12 and IL-15 immunocytokines are functional, and can be expressed in CAR NK cells thereby modulating the cells´ growth and direct antitumor activity as well as their stimulatory activity towards bystander immune cells.

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Development of a chimeric costimulatory receptor to enhance CAR T therapy

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The use of chimeric antigen receptor (CAR)-expressing T cells re-directed to specifically recognize and eliminate malignant cells, greatly increased the scope and potential of adoptive immunotherapy and is being assessed for new standard of care in certain human malignancies. However, therapies based on such gene-modified T cells, although sometimes efficacious, have a high potential for improvements regarding efficacy and safety. While CAR T cells have proven to be very efficacious in hematologic malignancies, their effectiveness in solid cancers has yet to be shown. Due to challenges presented by the immune-suppressive environment and challenges to infiltrate solid masses it is likely that novel strategies are required to ameliorate T cell function in such settings.
To this effect our work is focusing on strategies to enhance the CAR-based immune response in melanoma by co-expressing a CAR and a chimeric costimulatory receptor (CCR) in the same T cell. This two-receptor approach includes a second generation CAR specific for chondroitin sulfate proteoglycan 4 (CSPG4) and a CCR that recognizes CD20.
For this study, we have set up high-throughput cloning and in vitro experimental techniques that enable the rapid generation and functional testing of a multitude of CD20 CAR encoding lentiviral constructs which differ with regard to their co-activation and signaling domains. TransAct™ Reagent-activated T cells were lentivirally co-transduced with a CAR as well as a CCR. Subsequently, engineered T cells were enriched, expanded and co-cultured with either Mel526 cells ($CSPG4^+$) in the presence (or absence) of CD20 positive cells (B cells or JeKo-1) or gene-modified Mel526 cells expressing both CSPG4 and CD20. A specific release of pro-inflammatory cytokines and an increased killing confirmed a “boosted” immune response of the T cells co-expressing a CSPG4 CAR (4-1BB_CD3z) combined with either one of two CD20 CCR with different costimulatory sequences only in the presence of both targets CSPG4 and CD20. Altogether, this data supports the idea of using a CD20 CCR as a boosting tool in general. Current experiments are focusing on technical improvements and in vivo studies.

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Neoantigen-specific CD8 T cells are structurally and functionally overlapping with CD8 T cells recognizing shared self-tumor and viral epitopes

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Next-generation Adoptive Cell Transfer (ACT) therapy requires optimal methodologies to identify and isolate highly functional tumor-specific T cells or their T cell receptor (TCR). Recently tumor neo-antigens (neoAgs) resulting from non-synonymous somatic tumor mutations emerges as highly promising tumor rejection antigens and were associated to the clinical benefit of immunotherapy. The superior clinical relevance of neoAg-specific CD8 T cells over that of shared- self tumor-associated antigens (TAA) is hypothesized to be associated with their high affinity TCR since they escape central tolerance. Indeed, these might be of high interest for immunotherapy given that TCR-Peptide-Major Histocompatibility Complex (pMHC) structural affinity has shown strong correlations with CD8 T cell responsiveness. Lately, TCR affinity has been assessed thanks to the advent of reversible multimers (NTAmers), allowing measurement of monomeric pMHC-TCR dissociation kinetics on live CD8 T cells and also isolation of highly functional T cell clones. Here, we comprehensively characterized neoAgs-specific T cells and refined selection criteria for the isolation of highly functional tumor- specific CD8 T cells for ACT. Indeed, CD8 T cells directed against 3 viral epitopes (CMV, EBV and Flu), 5 TAA (MUC1, NY-ESO1, gp100, MAGE-A10 and Melan-A) and 5 neo-epitopes were identified and functionally profiled in-depth. We used soluble reversible pMHC multimers to sort “untouched” CD8 T cells and measure monomeric pMHC-TCR dissociation kinetics. Comprehensive analysis of functional and structural avidity as well as TCR sequencing were performed for individual clones. Our data revealed that, as compared to TAA-, viral- and neoantigen-specific CD8 T cells are more prone to apoptosis following soluble pMHC multimers activation. Moreover, using reversible as opposed to conventional pMHC multimers, the cloning efficiency and TCR diversity increased for viral epitopes but not for TAA and surprisingly only for some neoantigen specificities. Finally, consistently with the previous observations, comprehensive functional analyses showed that neoantigen-specific T cells cover a wide range of structural and functional avidity and partially overlap with that of viral epitopes and TAA. These results demonstrate that reversible multimers provide a valuable solution to reliably isolate highly functional cells, which is critical for next-generation ACT. Furthermore, comparison of neoAg-specific CD8 T cells with viral and TAA- specific ones revealed heterogeneous structural and functional avidities, arguing that not all neoAgs-specific T cells are equally attractive candidates for ACT, and that relevant filtering should be applied to identify the most relevant neoepitopes/TCR for mutanome-based immunotherapy.

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“Built-in” PD-1 Blockade: a strategy to counteract inhibition of effector cells for adoptive cell cancer therapy

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Tumor elimination depends on the ability of the immune effector cells to persist, proliferate and function optimally in the immunosuppressive tumour microenvironment. Although CAR and TCR based immunotherapies have shown great clinical potential, some challenges remain which jeopardize the efficacy of the treatment. Effector cells naturally become exhausted and upregulate inhibitory receptors enabling tumor cells to escape the immune radar by expressing inhibitory ligands. The PD1-PDL1 axis is the most investigated pathway. We here report an engineered immune effector cells which incorporate an intrinsic PD-1 blockade that modulates the inhibitory effect of PD1-PDL1 signaling. We validate our construct in two types of effector cells; the FDA approved NK-92 cell line, plain or engineered, and in primary Tc. We assessed the effect of intrinsic PD-1 blockade by measuring cytokine production, degranulation and cytotoxicity in effector cells upon encounter with PD-L1+ tumor. Preliminary data indicate that our innovation enhanced the effector functions of NK-92 cells against PD-L1+ target cells, suggesting that engagement of our PD-1 blocker with PD-L1 inhibits

the negative axis. Our results demonstrate the beneficial effect of providing effector cells with an intrinsic PD1-PDL1 blockade to counteract PD-1 exhaustion. Of importance, our PD-1 blocker did not interfere with TCR signaling and with target recognition when the target cells were PD-L1 negative. Potential advantages in using our PD-1 blocker in comparison to other strategies adopted to either block inhibitory PD-1 signal (blocking antibodies) or to convert it into co-stimulatory signal (PD1-CD28) are (i) PD-1 signaling do not modify TCR signaling; (ii) no systemic removal of PD1-PDL1 break reduces the risk of autoimmune toxicities; (iii) less cell exhaustion due to the presence of the costimulatory signaling domain (PD1-CD28). In conclusion, we present a method for increasing efficacy of T- or NK-cell based therapies and avoiding toxicity often seen in patients after antibody blockade of this pathway.

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T cells isolated from patients with checkpoint inhibitor resistant-melanoma are functional and can mediate tumor regression

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Background: The majority of patients with metastatic melanoma obtain only short-term or no benefit at all from checkpoint inhibitor (CPI) immunotherapy. In this study, we investigated whether the immune system of patients progressing following CPI treatment was able to generate functional tumor-specific immune responses.
Materials and methods: Tumor-infiltrating lymphocytes (TILs) were isolated and expanded from metastatic melanoma lesions which progressed during or after anti-PD-1 and anti-CTLA-4 treatment. Tumor-specific immune responses were assessed with co-culture assays of TILs and autologous tumor cells.
Results: TILs from 23 metastases of individual patients could be assessed for T cell recognition of autologous tumor cells. All metastases were progressive on or following anti-PD-1 (23/23), and the majority also after anti-CTLA-4 (17/23). Functional anti-tumor immune responses were detected in 19/23 patients (83%). Both $CD8^+$ (in 18/23 patients, 78%) and $CD4^+$ (in 16/23 patients, 70%) TILs were able to recognize autologous tumors. A large fraction of $CD8^+$ TILs (median 23%, range 1.0-84%) recognized tumor cells. This is similar to the cohorts of unselected patient populations with metastatic melanoma presented in previous studies. The localization of intratumoral immune infiltrates was heterogeneous among samples. In a phase I/II clinical trial, TILs were administered with lymphodepleting chemotherapy, pegIFNa2b and IL-2 to 12 patients with CPI-resistant melanoma. Out of 12 patients who previously failed CPI therapy, treatment with TILs resulted in two partial responses, of which one is ongoing.
Conclusions: Tumor-reactive T cells appear to heavily infiltrate the tumor microenvironment of patients who failed previous CPI treatment. These patients can still respond to an infusion of unselected autologous TILs. Our results warrant further testing of novel immune re-activation strategies in melanoma patients who failed multiple CPI therapy.

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Donor-specific immunomodulation by clinical MIC cell infusion - a phase I study (TOL-1)

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Long term immunosuppression after transplantation can cause life threatening side effects. Therefore, there is a fervent need to induce a donor-specific tolerance without broad unspecific immunosuppression. To this aim we performed a clinical phase I study with donor peripheral blood mononuclear cells treated by mitomycin C (MIC cells).
All ten transplant recipients received MIC cells manufactured under GMP conditions out of leukapheresis products from their kidney donors. The amount of MIC cells administered to the patient was escalated from 1.5 x 10^6 MIC cells per kg body weight at day -2 (N=3, group A), to 1.5 x 10^8 MIC cells per kg body weight at day -2 (N=3, group B) or on day -7 (N=4, group C) before transplantation. After transplantation, patients received a standard triple drug immunosuppressive therapy. The frequency of adverse events (AE) was measured as primary outcome at day 30.
All kidney transplant recipients showed a median serum creatinine of 1.4 mg/dL at day 30 and remained stable with a median creatinine of 1.48 mg/dL at day 180 without significant proteinuria (median 10g/mol creatinine at day 180) and without rejection episode. In total 70 AEs were observed including four severe AEs but not related to the MIC cell therapy. Besides two infectious complications, no other AEs such as positive cross match results, *de novo* donor-specific antibodies or rejection episodes were recorded. In group C, a reduction of immunosuppressive therapy was effective in the observational phase with low-dose cyclosporine A and low-dose enteric-coated mycophenolate sodium.
Immunologically, an increase of $CD19^+$ B cells up to a median of 300/µL until day 30 was observed followed by a decrease to a median of 35/µL at day 180 in group C. Notably, $CD19^+CD24^{high}CD38^{high}$ transitional Bregs increased from a median of 2% at day 30 to a median of 20% on day 180. The plasma ratio of IL-10/TNF-α increased from a median of 0.05 before cell therapy to a median of 0.11 at day 180. In an *in vitro* mixed lymphocyte culture assay patient lymphocytes showed no or only minimal reactivity against irradiated donor lymphocytes while reactivity against 3rd party lymphocytes was preserved.
Additionally the potency of MIC cell products was analyzed in *in vitro* assays. These demonstrated that MIC cells have the capability to induce tDCs with down-regulation of costimulatory molecules CD80 and CD86 and the maturation molecule CD83 and up-regulation of the immunosuppressive molecule CD103. Moreover, the immunosuppressive function of tDCs was confirmed by inhibition of IFN-γ release and proliferation of CMV-specific T cells.
A stable function was observed in all transplant recipients receiving the MIC cell product without any allograft injury nor rejection episodes even under reduction of conventional therapy with immunosuppressive drugs. MIC cells constitute a novel type of immunotherapy which has a high potential in transplantation medicine and also cancer therapy.

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Development of flow cytometric assays for CAR T cell manufacturing and patient immunomonitoring

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Adoptive cell therapy using genetically engineered chimeric antigen receptor (CAR) T cells has demonstrated unprecedented potency in B cell malignancies, and offers new hope for curative responses in patients suffering from cancer. However, the manufacturing process for CAR T cells is very complex and has extensive demands on personnel and infrastructure, which is a major obstacle for their routine clinical use. To overcome these hurdles, the CliniMACS Prodigy® allows generation of CAR T cells in a single automated and closed system, including initial cell washing, T cell enrichment, activation of the enriched T cells, lentiviral transduction, expansion, and final formulation for patient application.
For assessment of CAR T cells during cell manufacturing and patient immunomonitoring we developed a set of different flow cytometric assays. These assays will be used for 1) in-process control, QC release testing, and concomitant research during the manufacturing process, and 2) for determination of CAR T cell persistance and phenotyping during patient immunomonitoring. Among others these assays allow to determine the general immune cell composition, CAR transduction efficiency, and further functional CAR T cell phenotypes like differentiation, activation, or exhaustion status. All stainings feature a simple no-wash protocol and have been tested on leukapheresis and cultured CAR T cells for cell manufacturing, and on whole blood for patient immunomonitoring.
For identification of CAR T cells we developed CAR detection reagents that specifically bind to the antigen-recognition domain of the receptor. Thus, these detection reagents discriminate between various CAR constructs, and can be used for enumeration of CAR T cells during manufacturing and immunomonitoring.
For all flow assays mentioned above so-called Express Modes have been programmed, that allow an automated acquisition and analysis of stained samples on MACSQuant® Analyzers. These Express Modes feature predefined experiment settings and analysis templates, and apply a fully automated gating strategy that adapts for each individual data file. This allows for a high standardization by reducing operator variability, full reproducibility of data analysis, and future integration into automated workflows.
Elaborate flow assays specifically designed for CAR T cells, run with high-quality antibodies and fully automated flow analysis, provide a robust assessment of cell manufacturing and patient immunomonitoring. This will help with establishing complex individualized therapies and will allow us to understand from future clinical trials in greater detail the phenotypic changes occuring throughout the life time of a CAR T cell.

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Super-resolution microscopy *d*STORM reveals CD19^{dim} expression on a subset of myeloma cells that can be targeted with CD19-CAR T cells

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Background: Immunotherapy with chimeric antigen receptor (CAR)-engineered T-cells targeting CD19 (CD19CART) is being evaluated in multiple myeloma, a clonal proliferation of plasma cells. A recent study by Garfall *et al* reported complete remission in a patient that had received CD19CART even though only 0.05% of myeloma cells expressed CD19 as judged by flow cytometry (FC), the routine detection method. The mechanism for this response has remained unclear and sparked debate over low level CD19 expression on myeloma cells that may not be detectable by FC but trigger elimination by CD19CART.
Methods: We generated expression profiles of CD19 on myeloma cells from n=14 patients by single-molecule sensitive super-resolution microscopy (*d*STORM - *direct* stochastic optical reconstruction microscopy) and FC. In parallel, we treated myeloma cells with CD19CART and control T cells *in vitro*.
Results: In 10/14 patients, we detected CD19 on a fraction of myeloma cells (range: 10.3%-80%) by

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*d*STORM, while FC detected CD19 only in two out of these 10 patients on a smaller cell fraction (range: 4.9%-30.4%). Four patients were classified as CD19-negative by *d*STORM. The majority of myeloma cells expressed CD19 at very low levels, far below the FC detection limit. Treatment with CD19CART led to specific elimination of $CD19^{dim}$ myeloma cells, even when CD19 was undetectable by FC. The threshold for CD19CART recognition was below 100 CD19 molecules per myeloma cell.
Conclusions: In a prevailing subset of patients, CD19 is expressed on a large fraction of myeloma cells at a very low density, only detectable by super-resolution *d*STORM microscopy. These patients might be candidates for therapy with CD19 CART cells. Our data rationalize anti-myeloma responses that have been reported after CD19CART therapy. *d*STORM analysis allowed defining the threshold of antigen expression for T cell activation via a CD19 CAR, which was found to be less than 100 molecules per cell.

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Induction of tolerogenic dendritic cells *in vitro* by mitomycin C-induced peripheral blood mononuclear cells
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Background: Tolerogenic dendritic cells (tDCs) with immunomodulation function have been exploited as a potential cell-based therapy for organ transplantation and autoimmune diseases. Various *ex vivo* methods have been developed to generate stable tDCs that are able to induce and maintain immune tolerance. In our study, Mitomycin C-treated donor peripheral blood mononuclear cells (MICs) were used for generation of donor-specific recipient-derived tDCs *in vitro*. The immunomodulating potency of tDCs was determined by the following parameters: the phenotype of tDC, the immunosuppressive capacity of tDCs on allo-reactive $CD4^+$ and $CD8^+$ T cells as well as on antigen-specific $CD8^+$ T cells.
Methods: Third-party immature DCs (iDCs) were generated 3 days before MIC production. Each batch of MICs was introduced to 2 x 10^5 iDCs at different ratios for a two-hour interaction. Afterwards a DC maturation cocktail was added for overnight coculture. Then tDCs were purified by magnetic separation using Pan-DC Enrichment Kit. Characterization of tDCs was performed by checking the expression of MHC class II molecule HLA-DR, maturation marker CD83, co-stimulatory markers CD80 and CD86, as well as an immunosuppressive marker CD103 using flow cytometry. The immunosuppressive function of tDCs on allo-reactive T cells was determined by mixed lymphocyte reaction (MLR) assay. The secretion of IFN-γ by CMV-specific T cells was measured by an ELISpot assay. Moreover, the proliferation of CMV-specific T cells was evaluated by tetramer staining followed by one-week mixed lymphocyte peptide culture (MLPC).
Results: Comparing to mDCs, tDCs are characterized by low expression of co-stimulatory markers (CD80, CD86) and maturation marker (CD83) as well as high expression of inhibitory marker (CD103). Functionally, the proliferation of allo-reactive $CD4^+$ and $CD8^+$ T cells was hampered by tDCs *in vitro*. Inhibition of IFN-γ release and proliferation of CMV-specific T cells was clearly observed as well.

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In vitro expanded tumor-infiltrating lymphocytes across various sarcoma subtypes highly express LAG3 and comprise multifunctional T cells with tumor killing capacity
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Background: Tumor specific TILs can be in vitro expanded and have the ability to induce complete and durable tumor regression in some patients following ACT. In this preclinical study we investigated the feasibility of expanding TILs from sarcomas, as well as performing functional in vitro analyses on these.
Methods: Fresh tumor samples from sarcoma patients were obtained, and TILs were isolated and expanded in growth medium containing IL-2. Accumulating evidence supports that in vitro manipulation of the tumor microenvironment by addition of stimulatory antibodies can improve quality of expanded TIL. Thus, we also investigated the effect of adding an agonistic CD137 antibody (Urelumab, BMS) and/or an agonistic CD3 antibody (OKT3) to the growth medium. Phenotype and functional analyses were performed using flow cytometry and IFNγ-Elispot. Cytotoxicity analyses were performed using Xcelligence.
Results: We obtained fresh tumor samples from 28 patients with 8 different sarcoma subtypes, and we were able to expand a minimum of 40 million TIL from 25 of these (90%). Mean expansion times were 32 days (14 - 61) and expanded cells were predominantly ab T-cells (71 %) of effector memory subtype. T-cells had a mean CD8/CD4 ratio of 0.5 indicating an overrepresentation of CD4+ TIL. Especially CD8+ TIL highly expressed LAG3 and to a lesser degree PD-1 and BTLA. A sub study revealed that adding anti-CD137 and/or OKT3 increased total yield of TILs; anti-CD137 skewed the phenotype significantly towards more CD8+ TILs and in some cases NK cells and γδ cells.
TILs from 10 of 22 tested tumor samples from four different sarcoma subtypes (undifferentiated pleomorphic sarcoma, myxofibrosarcoma, myxoid liposarcoma and osteosarcoma) demonstrated reactivity against autologous tumor cells using IFNγ-Elispot (40 - 500 spots per 100.000 TILs). These results were verified in an intracellular cytokine release assay using flow cytometry and showed multifunctional capacity among the reactive TIL. In TILs stimulated with anti-CD137 the reactivity increased in four of four tested samples. Further analyses of agonistic antibodies on modulation of TIL expansion, phenotypes and biomarkers are ongoing.
Conclusion: We were able to expand TIL from 90 % of the acquired tumor samples to numbers needed for possible future clinical ACT implementation. Expanded TILs were a mix of CD4+ and CD8+ with CD4+ being predominant. CD8+ TIL highly expressed LAG3 and to a lesser degree PD-1 and BTLA. Approximately half of the TIL cultures showed some degree of in vitro tumor reactivity as determined by Elispot and flow cytometry. Early analyses suggest that addition of anti-CD137 could influence expansion time, phenotype, and functional capacity of the expanded TILs. Based on these results, we conclude that it is feasible to translate TIL based ACT into clinical testing in sarcoma patients.

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Ex vivo generation and expansion of CAR-engineered NK cells for adoptive cancer immunotherapy

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Natural killer (NK) cells hold promise for adoptive cancer immunotherapy. Like T cells, the antitumor

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activity of NK cells can be enhanced by expression of chimeric antigen receptors (CARs) that facilitate selective recognition and antigen-specific lysis of target cells, hence bypassing the need for activation of endogenous cytotoxicity receptors. For adoptive immunotherapy, NK cells are usually isolated from peripheral blood (PB) and expanded ex vivo with cytokines before infusion into patients. Experimentally, NK cells have also been derived from hematopoietic stem cells (HSCs) by ex vivo differentiation following different protocols. CAR-NK cells may be generated from CAR-gene transduced HSCs following a similar approach.
To explore this strategy, we established a cytokine-based protocol for ex vivo expansion and subsequent differentiation of PB-derived $CD34^+$ HSCs into functional $CD56^+$ NK cells. When the $CD34^+$ cells were first transduced with an ErbB2 (HER2)-specific CAR-encoding lentiviral vector and then cultured following this protocol, they gave rise to ErbB2 CAR-expressing NK cells, albeit at low frequency. Nevertheless, the ex vivo generated CAR-NK cells were functionally active, displaying enhanced cytotoxicity against ErbB2-expressing tumor cells. To aid further expansion of NK and CAR-NK cells, we generated gene-modified K562 feeder cells that express different membrane-bound pro-NK cell factors including IL-15 and IL-21. Co-culture of PB mononuclear cells with these feeder cells allowed high and selective expansion of NK cells over time, demonstrating efficacy of this approach. In pilot experiments, addition of gene-modified K562 feeder cells to ex vivo differentiating $CD34^+$ cell pools also promoted development and marked expansion of functional NK cells. Likewise, feeder cell-mediated expansion of ex vivo generated CAR-NK cells is being explored.

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CD19 splicing and mutation linked to CART-19 immunotherapy resistance

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CART-19 immunotherapy utilizes chimeric antigen receptor-armed T cells (CART-19) to target the CD19-antigen on B-cell acute lymphoblastic leukemia (B-ALL) blasts. Different trials in the US have shown promising results and therefore CART-therapy was approved by the FDA in 2017. Nevertheless, relapse under therapy occur in up to 20% of the pediatric patients. Previous works suggests that the relapse may be attributable to a preexisting splicing variant of CD19 lacking the exon 2 and therefore the epitope targeted by CART-19. We recently published that an alternatively spliced CD19 mRNA isoform lacking exon 2 is expressed at different levels in leukemic blasts at diagnosis in children and in the bone marrow of non-leukemia-pediatric donors. These results prove that some of the CD19 isoforms contributing to CART-19 escape already pre-exist at diagnosis and could evolve as a dominant clone during CART-19 therapy. In this project, we propose to dissect the molecular determinants of CD19 exon 2 splicing.
We investigated the CD19 locus with NGS amplicon-sequencing to identify polymorphisms and mutations that could influence the CD19 splicing. The effects of the located mutations were further investigated with special interest to their position regarding different binding motifs for splicing factors. So far we screened 21 samples of 15 patients with 12 samples taken at initial diagnosis and 9 samples after complete remission. We identified several mutations on and upstream of the CD19 locus. 13 of the mutations we identified were only observed in the samples taken at diagnosis and are therefore linked to aberrant cells. Some of these mutations affected predicted motifs of RNA-binding proteins.
Our approach will identify mutated regions in the CD19 locus possibly affecting the splice of exon 2. In the future we plan to confirm the relevance of these positions by down regulation of the identified splice factors and by analyzing the identified mutations in patients relapsed under CART-19 therapy. This may help in the early identification of patients who will not benefit of a CAR 19-therapy.

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T Cell Receptor/DAP10 chimera as a means to attaining persistent anti-tumor T cell response
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Adoptive T cell transfer (ACT), the introduction of autologous tumor-specific T cells, has been proven to be a promising therapy against melanoma. However, the limited survival of tumor-infiltrating lymphocytes (TIL) after ACT poses a problem for anti-tumor efficacy. Our studies have shown that signaling through the stimulatory receptor NKG2D prevents cell death while enhancing anti-tumor potency and in vivo persistence in T cell Receptor (TCR)-transduced CD8+ T cells. In an effort to overcome post-ACT limited cell survival, we propose a novel approach: the coupling of TCR ligation to NKG2D signaling. For this, a tumor-reactive TCR will be fused to the signaling domain of DAP10 (TCR/DAP10 chimera), the adaptor molecule that mediates NKG2D signaling in CD8+ T cells. Human T cells expressing TCR/DAP10 will be examined for their signaling pathways, capacity to survive and ability to mediate the regression of an established human cancer using a humanized model of melanoma. We hypothesize that the NKG2D stimulatory pathway, as well as downstream TCR signaling, will be activated upon engagement of the TCR/DAP10 chimera, improving the survival of T cells after ACT. Our innovative strategy will allow us to carefully evaluate the intricate signaling pathways utilized by the hybrid TCR/DAP10. Potentially, these unique signals will enhance the survival of transferred T cells and mediate cancer regression.

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Optimal-affinity T cell receptors targeting NY-ESO via MHC II help MHC I-restricted T cell receptors in tumour regression
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Adoptive transfer of T cell receptor (TCR)-engineered T cells is a promising approach in cancer therapy but needs improvement for more effective treatment of solid tumours. While most clinical approaches have focussed on CD8 T cells, the importance of CD4 T cells in mediating tumour regression has become apparent. Regarding shared (self) tumour antigens, it is unclear, whether the human CD4 T cell repertoire has been shaped by tolerance mechanisms and lacks highly functional TCRs suitable for therapy. Here, TCRs against the tumour-associated antigen NY-ESO were isolated either from human CD4 T cells or from mice that express a diverse human TCR repertoire with HLA-DRA/DRB1*0401 restriction and are NY-ESO-negative. NY-ESO-reactive TCRs from the mice showed superior recognition of tumour cells and higher peptide sensitivity compared to TCRs from humans. We identified a candidate TCR, TCR-3598_2, which was expressed in CD4 T cells and caused tumour regression in combination with NY-ESO-redirected CD8 T cells in a mouse model of adoptive T cell therapy. These data suggest that MHC II-restricted TCRs against NY-ESO from a non-tolerant non-human host are of optimal affinity and that the combined use of MHC I- and II-restricted TCRs against NY-ESO can make adoptive T cell therapy more effective.

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Heterogeneity and dynamics of the tumor-infiltrating lymphocyte repertoire in melanoma and pancreatic cancer patients

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The presence of tumor-infiltrating lymphocytes (TIL) is associated with prolonged survival in many cancers, and harnessing of the T-cell response through checkpoint inhibition or infusion of ex vivo expanded TILs can result in tumor regression.
We characterize the TIL repertoire of patients with pancreatic ductal adenocarcinoma and melanoma by T-cell receptor (TCR) deep-sequencing, as well as phenotypic and functional analysis of bulk and single-cell expansion cultures.
Enrichment of highly frequent CDR3 sequences within TIL suggests in situ proliferation in response to tumor-derived antigens.
Intra-tumoral and intra-patient heterogeneity is significant and highly individual: TIL repertoires from multiple regions of the same tumor show an overlap between 8.4-100%. TILs from multiple lesions within the same patient share between 0-70% of TCRs and tend to overlap less if biopsies are not acquired concurrently, indicating a continuous turn-over or reshaping of TIL composition. Notably, repertoire-sharing is always most prominent among the largest TIL clones, possibly explained by efficient migration/re-circulcation of some clones, or their maintenance by ubiquitously expressed (tumor-)antigens.
Importantly, TIL repertoire composition undergoes drastic shifts during in vitro expansion, resulting in loss of dominant clones and enrichment of bystander clones with high proliferative potential.
Our findings call for careful sampling and optimized culture conditions for TIL infusion products and illustrate the need to probe T-cell reactivity directly ex vivo. The heterogeneity of the TIL response in cancer patients implies that therapeutic efficacy of TCR gene therapy using tumor-dominant TCRs could be more consistent than that of TIL therapy.

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Immunotherapy of ovarian cancer by targeting Claudin-6 with CAR-engineered T cells

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Adoptive transfer of chimeric antigen receptor (CAR)-engineered T cells is emerging as a promising approach for cancer immunotherapy and has shown impressive anti-tumoral activity in early clinical trials. As CARs are highly potent in mediating tumor cell-killing, precise targeting as well as absence of the target in healthy tissue is of seminal importance. Indeed, identification of cancer cell-specific targets is a limiting factor for CAR-based applications in solid tumors.
Claudin-6 (CLDN6), a member of the tetraspanin protein family, is involved in the formation of primitive tight junctions during organogenesis and therefore, exclusively expressed at significant levels during fetal development. Its absence in adult tissues, but overexpression in various high medical need cancers including ovarian, endometrial, testicular, lung and rare pediatric brain cancers, positions

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CLDN6 as ideal target for CAR-engineered T cells.
To develop a CLDN6-targeting therapy, three different CAR scaffolds were designed that all share the same scFv fragment conferring exclusive specificity to CLDN6. Among two different 2nd generation CARs, one with a CD28 (CLDN6-CAR-28z) and one with a 4-1BB (CLDN6-CAR-BBz) costimulatory domain, a highly innovative combinatory CAR format (comb. CLDN6-CAR) was evaluated for lead structure selection. The latter CAR scaffold is designed to be dependent on assembly and signaling of the endogenous CD3 chains and thus should lead to a more physiologic T-cell signaling.
T cells armed with the different CLDN6-CARs by retroviral gene transfer were extensively characterized *in vitro* regarding their potential to mediate antigen-specific effector functions. While all three CLDN6-CARs successfully mediated effector functions in short-term assays, qualitative differences emerged, if more complex assays were applied including repetitive killing of tumor cells using 3D cultures to determine long-term functionality and persistence. Especially, sequential tumor rechallenge served as a prediction tool for anti-tumoral efficacy *in vivo*. And in fact, those CLDN6-CARs exhibiting an enormous ability for repetitive killing *in vitro* also mediated impressive anti-tumoral efficacy *in vivo* using an advanced ovarian carcinoma xenograft model.
Hence, the most promising two CAR candidates, CLDN6-CAR-BBz and comb. CLDN6-CAR, were selected for preclinical development. A robust and cost-effective GMP manufacturing process applicable to both CAR candidates has been developed and is currently further optimized.

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Update on the clinical trial preparations concerning a MAGE-A1-specific T cell receptor

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Previously, we reported the isolation of an optimal-affinity TCR, T1367, specific for the cancer/testis antigen MAGE-A1 (Obenaus, et al. 2015). T cells, transduced with T1367, showed high activity against MAGE-A1$^+$ tumor cells *in vitro* and in an animal model with no notable off-target toxicity.
In cooperation with the hematology department of the Charité a phase I/IIa clinical trial was initiated to evaluate the efficacy of adoptive T-cell therapy with T1367 T cells in patients with relapsed/refractory multiple myeloma (r/r MM). In Q1 2018, we received positive approval of the Ethics-Committee of Berlin and the clinical trial authorization by Paul-Ehrlich-Institute.
The trial is designed as a classical 3+3 dose escalation study with doses of 10^5-10^8 T cells/kg bodyweight being administered after a lymphodepleting regimen with Cy/Flu. The primary endpoint will be safety of T1367 T cells assessed at day 84. We have screened n=136 bone marrow biopsies from MM patients treated at the Charité and found MAGE-A1 expression in 30% of the patients of which 40% have high expression levels. These patients will be eligible to be included in the study.
Further, we established a GMP-compliant manufacturing process for T1367 TCR-modified T cells at the Experimental and Clinical Research Center of MDC and Charité. This process enables us to transduce T cells with GaLV-pseudotyped gamma-retroviral vectors, reaching transduction rates of up to 60% in healthy donor material with a controlled mean vector copy integration number per transduced cell of less than 5. We could expand the cells up to 1x10^{10} CD8$^+$ cells with the majority of the cells exhibiting a stem cell memory phenotype. Further, the T cell product showed a comparable efficacy profile in the absence of toxicity *in vitro* compared to the T cells analyzed in preclinical studies.
The manufacturing license is expected to be issued in Q2 2018.
The preparations for the monitoring of the patients are ongoing.

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Off-target toxicity risk evaluation for TCR-based therapies guided by immunopeptidomics data of a comprehensive set of more than 650 primary normal tissue samples

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Targeting tumor-associated peptide:HLA complexes (pHLA) with T-cell receptors (TCRs) or TCR-like antibodies has evolved as a promising strategy in cancer immunotherapy. However, due to inherent degeneracy of such pHLA binding agents regarding epitope recognition, off-target cross-reactivity to peptides presented on normal tissues is a major concern and has indeed proven to be a substantial safety risk in previous clinical trials. Thus, a thorough assessment of TCR cross-reactivities is needed during pre-clinical validation of candidate TCRs. Depending on target and binder properties, *in silico* epitope prediction tools may generate extensive lists of candidate peptides, few of which will ever be presented by HLA molecules on healthy tissue. The value of such prediction tools for experimental validation strategies hence depends on a well-grounded rationale for similar peptide selection and ranking.
We analyzed the immunopeptidome of > 650 primary human specimens from > 40 normal tissue types by highly sensitive quantitative liquid chromatography - mass spectrometry (LC-MS). This broad, unparalleled data basis allows us to efficiently prioritize peptides for off-target toxicity assessment: Sequence-based characteristics like similarity to the target peptide are combined with experimental measurements for peptide presentation by LC-MS and RNA-Seq-based gene expression data, facilitating the rational selection and ranking of similar peptides for *in vitro* TCR cross-reactivity testing. We exemplarily show for a highly tumor-specific target how we perform cross-reactivity analyses during TCR candidate characterization at two steps with different focus: at an early developmental stage, similar peptides are searched based on the target peptide sequence and used to test for broadly cross-reactive TCRs that can thus be de-selected early on. At this stage, a representative set of critical, naturally HLA-presented peptides with high sequence identity or similarity to the target peptide is selected. At a later, safety-centric step, clinical candidate TCRs are comprehensively challenged with similar peptides based on TCR binding motifs as determined by positional scanning. Among these, peptides naturally presented on normal tissues are of highest safety relevance and either directly lead to TCR de-prioritization or are selected for TCR specificity assays with high priority. In summary, selection and ranking of candidate peptides based on their natural occurrence on a comprehensive set of normal tissues is a unique and exceptional feature of our off-target cross-reactivity evaluation process, enabling an efficient and well-informed safety approach for pHLA binders.

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TCR-pMHC 2D affinity and bond lifetime under force correlate with tumor-specific CD8 T cell responsiveness

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Monomeric pMHC-TCR dissociation kinetics measured with reversible pMHC multimers (NTAmers) on live tumor-specific CD8 T cells have been shown to correlate to T cell responsiveness within a range of physiological affinities. However, isolation of the most effective tumor-specific CD8 T cells for

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adoptive T cell therapy remains difficult to achieve, in part due to i) the low TCR affinity of self-tumor antigen-specific clones and ii) the high TCR affinity of some neoantigen-specific CD8 clones displaying exhausted phenotypes. Using the highly sensitive 2D micropipette adhesion frequency assay to measure 2D affinity and the biomembrane force probe (BFP) to measure bond lifetime under force, we were able to probe a panel of primary CD8 T cells transduced with sequence-optimized HLA-A0201/NY-ESO-$I_{157-165}$-specific TCR clones of incremental 3D affinities as measured by SPR. All clones tested displayed a catch bond phenotype under force indicative of productive antigen recognition. Whereas off-rates measured with NTAmers correlates with T cell function only within the physiological range of affinities, 2D affinity and bond lifetime under force strongly correlate with CD8 T cell functionality, even for supraphysiological TCRs. While 2D affinity and force measurements provide a more comprehensive analysis of CD8 T cell functions, the NTAmer technology remains a more high-throughput and sensitive approach and a valuable tool to identify rare high-affinity tumor-specific T cell clones. Our efforts are now focused on understanding the CD8 coreceptor contribution and the refinement of the selection of highly tumoricidal CD8 T cells.

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Modifying melanoma immune microenvironment by heterologous prime-boost vaccination with adenovirus and modified vaccinia Ankara virus vectors

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Introduction: Multiple vaccine dosing is usually necessary to prime and boost humoral and cellular immune responses against the target antigen. Administration of the same antigen by using different viral vectors termed heterologous prime boost has been experimentally applied against infectious diseases such as HIV, malaria, tuberculosis and certain malignancies such as prostate cancer. Here we have developed a novel immunization strategy against melanoma consisting of adenovirus and Modified Vaccinia Ankara (MVA) mediated heterologous prime-boost vaccination towards gp100 and TRP1 melanoma antigens coupled with adoptive cell transfer (ACT) of transgenic CD8+ and CD4+ T cells targeting the respective antigens.
Materials and methods: MVA and adenovirus vectors were engineered to ectopically express GP100 (CD8 T cell) and TRP1 (CD4 T cell) epitopes fused to mCherry reporter (MVA-PMTP and AD5-GTY, respectively). The functionality of the vectors was first tested *in-vitro* by co-culturing gp100 and TRP1 transgenic CD8+ and CD4+ T cells with transduced mouse splenocytes followed by IFNγ ELISA. Immunizations against established HCmel12 melanomas and in healthy B6 mice were carried out by priming the expansion of transferred CD4+ and CD8+ T cells with Ad5-GTY followed by MVA PMTP mediated boost 14 days later. CRISPR-Cas9 technology was used to generate Trp1 knockout HCmel 12 melanoma cell lines.
Results: Ad5-GTY and MVA-PMTP were able to stimulate PMEL CD8+ T cells and TRP1 CD4+ T cells *in vitro*. Both cell populations expanded efficiently in tumor free mice after Ad5-GTY priming. T cell expansion was efficiently boosted in the contraction phase with MVA-PMTP immunization in healthy but not HCmel12 melanoma bearing mice, where only PMEL CD8+ T cell expansion was observed. Interestingly, intra-tumoral route of administration of MVA booster vaccine was found to be therapeutically more efficacious than intra-peritoneal route in CD4+ T cell monotherapy suggesting local, beneficial effects in the tumor microenvironment. Trp1 knockout HCmel 12 melanomas were resistant to Trp1 CD4 T cell therapy indicating that CD4 T cell therapy requires target antigen expression by the melanoma cells.
Discussion: We have successfully generated adeno- and MVA vectors for priming and boosting of CD4 and CD8 T cell responses, respectively. Our results indicate that CD4+ and CD8+ T cell

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responses can be boosted in absence of melanoma, but in the tumor bearing hosts TRP1 CD4+ T cell boosting was inhibited possibly as a result of tumor induced changes in the phenotype of the transferred TRP1 CD4+ T cells.

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How to make a fishing rod for adoptive cellular therapy: the ACTolog® approach, a multi-targeted endogenous T-cell therapy

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Adoptive cellular therapy (ACT) is one of the major drivers currently revolutionizing cancer immunotherapy. Immatics in collaboration with the MD Anderson Cancer Center, USA (MDACC) developed a novel personalized ACT approach which is currently being tested in a phase I clinical study. Our approach is based on the pioneering studies of transfusion of isolated and ex vivo expanded endogenous tumor-specific T cells by Dr. Cassian Yee and colleagues at MDACC. Utilizing our proprietary target discovery platform XPRESIDENT®, Immatics has created a warehouse of multiple novel cancer targets. From this pool, the most suitable targets for each patient's tumor are identified and up to four personalized T-cell products are then manufactured for each patient under cGMP conditions. Expression of target source genes in a tumor biopsy is assessed by a quantitative polymerase-chain-reaction (qPCR)-based assay. If the tumor is positive for at least one warehouse target, a leukapheresis is taken from the patient for isolation of PBMCs. CD25 depleted PBMCs and antigen loaded autologous dendritic cells are then used for T-cell priming in vitro. Antigen-specific T-cells are sorted using in-house produced pHLA multimers and the Miltenyi MACSQuant® Tyto™ sorter. Following ex vivo expansion, the final T-cell product is cryopreserved in bags and stored until patient infusion. Multiple quality controls covering the entire manufacturing process and release testing ensure a safe and high-quality drug product. Here, we want to focus mainly on the generation and quality control mechanisms of the tools used for the identification and isolation of target-specific CD8+ T cells. By combining T cells specific for several targets, escape due to tumor heterogeneity should be limited. For immunotherapies, a multi-target approach is assumed to result in a broader immune cell invasion and stronger immune response against the tumor.

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PI3Kδ inhibition by idelalisib for optimized generation of CD19-specific chimeric antigen receptor T cells in chronic lymphocytic leukemia patients

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Anti-CD19 chimeric antigen receptor T (CART) cell therapy is currently among the most encouraging treatment approaches in cancer immunotherapy for B cell malignancies. However, CART cell generation is not standardized and may benefit from additional improvement, especially in leukemic patients. Particularly less-differentiated T cells are crucial for cell expansion, long-term persistence and therapeutic efficacy of CART cells. We investigated the effect of PI3Kδ inhibition with idelalisib during CART cell production. The PI3K/AKT/mTOR pathway is one of the main pathways involved in T cell differentiation. Peripheral blood mononuclear cells (PBMCs) of ten healthy donors (HDs) and six

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chronic lymphocytic leukemia (CLL) patients were transduced with a 3rd generation CD19.CAR.CD28.CD137zeta retroviral vector. PBMCs were activated with anti-CD3/anti-CD28 antibodies under addition of IL-7/IL-15. Cultivation was performed with or without 1 µM idelalisib. Viability, transduction efficiency, immune phenotype and cytokine production were analyzed longitudinally by flow cytometry. Cytotoxicity was evaluated via chromium-51 release assay. Idelalisib significantly increased the viability of CART cells derived from HDs (day 14: 86±5% vs 73±14%; p=0.009) and had a trend towards higher viability in CART cells derived from CLL patients (day 7: 92±3% vs 86±4%; p=0.005). Cell expansion was not impaired by idelalisib. Gene transfer efficiency in HDs (65±13% vs 60±12%; p=0.001) and in CLL patients (73±12% vs 67±14%; p=0.01) significantly increased with idelalisib on day 14. In CLL patients, T helper cells (CD3+/CD4+) decreased (day 14: 50±14% vs 71±12%; p=0.002) and cytotoxic T cells (CD3+/CD8+) increased (day 14: 45±15% vs 24±11%; p=0.002) with idelalisib. This CD4:CD8 ratio closer to 1:1 is considered to be more beneficial for engraftment and cancer eradication. PI3Kδ inhibition increased naïve-like T cells in all CART cells from HDs (31±11% vs 23±9%; p< 0.001) and in cytotoxic CART cells from CLL patients (19±14% vs 8±6%; p=0.04) on day 14. Idelalisib significantly reduced expression of the exhaustion markers Tim-3 and PD-1 in HDs (66±10% vs 82±5%; p=0.003 and 4±3% vs 6±3%; p=0.003) and in CLL patients (62±11% vs 72±17%; p=0.05 and 30±10% vs 41±14%; p=0.009) on day 14. Cytotoxic lysis was similar in both conditions. After stimulation with CD19+ Daudi cells, intracellular TNF-α (50±8% vs 64±8%; p< 0.001) and IFN-γ production (35±14% vs 46±14%; p< 0.001) significantly decreased in presence of idelalisib in CART cells from HDs. A similar trend was observed in CLL patient cells. This effect was reversible after overnight resting without idelalisib. In summary, *ex vivo* treatment with the PI3Kδ inhibitor idelalisib generated less-differentiated and less exhausted CART cells. In CLL patients, idelalisib led to a probably more beneficial CD4:CD8 ratio. Optimized CART cell generation will further enhance the clinical success of this encouraging treatment approach.

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Enhancing cancer immunotherapy using responsive biomaterials

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Adoptive cell therapy (ACT) employing antigen-specific T-cells has elicited dramatic clinical responses in leukemia and a subset of melanoma patients. However, strategies to safely and effectively augment T-cell infiltration and function in solid tumors remain of great interest. Our laboratory aims to enhance adoptive T-cell therapy and other cancer immunotherapies through responsive nanoparticle drug delivery. Here we describe a strategy to enhance the tumor-infiltration and function of transferred T-cells by spatiotemporally controlled delivery of immunomodulators. Responsive protein nanogels (NGs) containing large quantities of immunomodulatory drugs are designed and synthesized to release the drugs in response to the reductive environment specific in tumor tissue or on T-cell surface. We show that T-cells increase their cell surface reduction potential upon activation, which we exploit through the design of cell surface-bound NGs that disassemble to release protein cargos in response to this change in the local reductive environment following T-cell receptor (TCR) triggering. The T-cell surface-bound NGs selectively release adjuvant drugs in response to TCR activation, focusing drug release in sites of antigen encounter such as the tumor microenvironment. Using an IL-15 superagonist complex as a candidate adjuvant drug cargo, we demonstrate that relative to systemic administration of free cytokines, NG delivery selectively expands adoptively transferred T-cells 16-fold in tumors, and allows at least 8-fold higher doses of cytokine to be administered without toxicity, leading to substantially increased anti-tumor efficacy and safety. This strategy provides a general approach to augment the function of cell therapies by linking drug release to cell function in vivo.

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Intestinal graft-versus-host disease is driven by BATF-dependent IL-7R^{hi}GM-CSF^{+} T cells

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Acute graft-versus-host disease (GVHD) represents a severe, T cell-driven inflammatory complication following allogeneic hematopoietic cell transplantation (allo-HCT). GVHD often affects the intestine and is associated with a poor prognosis. Although frequently detectable, the role of the pro-inflammatory IL-17a-producing T helper lymphocyte subset (Th17) in the pathogenesis of intestinal GVHD is controversially discussed and remains to be fully elucidated. In this study, we addressed the functional role of the Th17-defining transcription factors such as BATF (Basic leucine zipper transcription factor ATF- like).
First, we performed gene expression analyses of transcription factors in GVHD-affected colonic tissues from mice and men undergoing allo-HCT and found high expression of BATF in comparison to unaffected tissues. Given that BATF is predominately expressed by lymphoid lineages, we hypothesized that BATF-expressing T cells might contribute to this enhanced expression. To address this, *Batf*$^{-/-}$ donor T cells in comparison to WT donor T cells were adoptively transferred in both complete MHC and miHA-mismatched GVHD models. These in vivo GVHD-experiments demonstrate that BATF is indispensable for the manifestation of intestinal GVHD. We found initial expansion and homing of allo-reactive T cells into the colonic lamina propria (cLP) compartment to be largely unaffected in the absence of BATF. However, upon GVHD onset the magnitude of the colonic donor T cell population became increasingly BATF-dependent. Interestingly, in addition to Th17 differentiation, we found that T cell-intrinsic GM-CSF- and IL-6-expression of colonic donor T cells were highly dependent on BATF while Th1 differentiation was unaffected. Functional studies employing cytokine reconstitution experiments of *Batf*$^{-/-}$ donor T cell-receiving mice and cytokine inactivation studies by using *Csf2*$^{-/-}$ donor T cells clearly supported that GM-CSF is promoting GVHD-associated colitis manifestation. GVHD was suppressed by a combined IL-7R and GM-CSF blockade, thereby suggesting synergistic mechanisms to inhibit the functionality of intestinal IL7R^{hi}GM-CSF^{+} donor T cells. Finally, reconstitution studies functionally demonstrated that intestinal GVHD-derived IL-7R^{hi} cLP T cells were able to reconstitute GVHD-associated colitis in *Batf*$^{-/-}$ donor T cell receiving mice. Together, this study provides a crucial example of a BATF-dependent, however Th17 fate-independent regulation of a pathogenic effector T cell population with critical relevance for intestinal GVHD. Hence, therapeutic targeting of the IL-7R/BATF/GM-CSF-axis might represent a novel future option to mitigate acute, life-threatening intestinal GVHD.

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Enhancing the activation and releasing the brakes of NK cells for treatment of Multiple Myeloma

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Natural Killer (NK) cells are innate lymphocytes with a strong anti-tumor ability. In multiple myeloma (MM) patients, an elevated number of NK cells after stem cell transplantation (SCT) seems to correlate with a higher overall survival rate. With the aim of NK cell use for adoptive cell therapy, we investigated the activation status and cytotoxic capacity of patient-derived NK cells at first diagnosis, before and after autologeous SCT. At all addressed time points, we observed significant changes in NK cell phenotype as well as cytotoxic function before and after cytokine-induced ex vivo expansion. Remarkably, after cytokine stimulation, patients NK cells showed a highly activated phenotype and significantly enhanced killing capacity of different types of MM cells that was comparable to healthy donors NK cells. In a small cohort of MM patients, we were able to isolate both NK cells and autlogeous MM cells from the bone marrow (BM). Interesting, BM-NK cells were able to kill MM cells as efficiently as patients PB NK cells. In parallel, we could show that PB-NK cells expanded from the same patient, were able to gain in lytic capacity against autologous tumor cells, suggesting a potential use of ex vivo activated autologeous NK cells as adoptive therapy for MM patients. With the goal to further improve the NK cell killing capacity against MM cells, we investigated the potential use of NK specific check point inhibitors with focus on NKG2A because this inhibitory NK cell receptor was upregulated following ex vivo cytokine stimulation and MM cells showed HLA-E expression that could even be increased by exposure to IFN-g.
Importantly, the blocking of NKG2A resulted in a significant increase in the NK cell-mediated lysis of all different MM target cells tested, including the primary autologous MM cells.
Finally, these results let suggest that combining cytokine induced NK cell activation and the specific check point inhibition of the NKG2A-mediated pathways can be an effective strategy to optimize NK cell therapeutic approaches against multiple myeloma.

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Combining conventional therapy with immunotherapy: a risky business?
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It has been well established that the immune system is an important player in the onset and development of cancer. This mechanism arises in every tumor, but the predominant players in the immune suppressive microenvironment are different for each cancer, possibly even for different cancer stages. This tumor immunologic knowledge has been explored insufficiently, certainly in gynaecologic malignancies. Nevertheless, this information is crucial when we want to implement immunotherapy that will change this environment. Moreover, there is sufficient evidence that the conventional therapies, like chemotherapy and radiotherapy also have their impact on this immune balance. Therefore, it may become critical to know early on what the status of the immune system is, before we start combinations. At this moment, we lack preclinical data to propose the most optimal combinations and dosages.
In our own experience with the ID8 serous ovarian cancer mouse model, we were able to shorten overall survival if combination regimens were altered. If we administered immunotherapy first (in this experiment, immunotherapy was given by means of dendritic cell (DC) immunotherapy) and then chemotherapy (carboplatin-gemcitabine), mice died earlier compared to the regimen if chemotherapy and immunotherapy were administered simultaneously. In another series of experiments in this mouse model, we could demonstrate that one week after administration of carboplatin-gemcitabine, a significant increase in circulating monocytic MDSC, Treg and macrophages and a decrease in $CD4^+$ T cells was present. This underlines the fact that we need to carefully select chemotherapy regimens for combination with immunotherapy.
Immunotherapy and its combinations certainly have a place in the current cancer therapeutic field, but

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timing, dosage, the choice of compounds and their combinations hold the key to success and this information is lacking these days. We therefore plead for thorough preclinical work, serious investment in immune monitoring and read out methods for early response. We cannot permit ourselves to find the right combinations by trial and error as was done in the early 1980s whereby countless women had to die of toxicity or cancer before a suitable treatment schedule was found. If we want to successfully include immunotherapy in current practice, we need to cleverly design the trials to obtain a benefit. If we fail, immunotherapy will only be known for its side effects and not for the success stories.

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A two chain chimeric antigen receptor (CAR) scaffold recruits T-cell endogenous CD3 subunits for physiological CAR signaling and induces efficient tumor cell killing *in vitro* and *in vivo*

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With the recent approval of the first chimeric antigen receptor therapies targeting CD19 in hematological malignancies, the CAR field has received further attention. The broadly applied CAR design is based on a single-chain fusion of antibody based antigen recognition domain fused to the CD3ζ chain followed by cytoplasmic co-stimulatory domains such as CD28, 41-BB or others. It is well known that the type of CAR scaffold not only determines the redirection of the immune reactivity against the chosen antigen, but also influences strength and quality of signaling thereby modulating T cell expansion and persistence as well as the strength of T cell activation. We are working on alternative antigen receptor designs for a more physiological T-cell activation. By hooking an antibody variable domain onto T-cell receptor invariant Cbeta-domain and co-expression with Calpha, we achieved a recruitment of the CAR/CD3-complex to the T-cells' surface. A first version of this prototype required the murine Calpha/beta constant domains as a prerequisite for a stable assembly thereby providing a risk for host immune responses against the mouse domain that can result in adverse events or eradication of CAR positive T-cells.
To address this risk and for further improvement, we redesigned our CAR in order to i) skip murine C-domains and to ii) increase the valency of antigen binding. To exploit the strong interaction between VH and VL domains, we connected either VH- or VL-domains as duplicates in series on either Calpha- or Cbeta-chain. Conceptually, this strategy should provide sufficient interchain interaction while using human C-domains. Of note, the so-called “combinatory CAR” binds its antigen strictly by engagement of independent receptor chains, not, as it is the case for classical CARs, within a single polypeptide chain. Anti-CAR idiotype staining confirmed that it can be expressed on CD8 positive T-cells after IVT RNA electroporation and that the antigen binding site of the CAR was intact.
Strong and specific T-cell responses were initiated upon antigen encounter *in vitro*. Compared to a 2nd generation single chain CAR the combinatory CAR showed superior immune activation, cytokine release, higher cytotoxicity and improved T cell proliferation at low surface antigen density on tumor cells. In a tumor spheroid based cytotoxicity assay, we observed a faster killing of antigen positive tumor nodules *in vitro*.Together, the high avidity and quick killing might represent a decisive factor in timely clearance of a tumor lesion with heterogenous antigen expression.
Finally, we validated strong anti-tumoral effects of combinatory CAR transduced T-cells in a xenograft mouse model. The T-cells were able to eradicate solid tumors in an early (10mm^3) as well as advanced (tumor size 300mm3) engraftment model.

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Depletion of macrophages in the tumor-draining lymph node enhances dendritic cell-induced anti-tumor immunity

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Background and Research Question: Dendritic cell (DC) vaccination has been proven a safe and effective treatment option for various cancer types, including malignant mesothelioma. Although promising results have been obtained with DC-vaccination, effectivity is thought to be hampered by immunosuppression induced by tumor cells or suppressive immune cells in the tumor microenvironment. Recently, it was shown that depletion of macrophages improved DC-vaccination efficacy in multiple intraperitoneal (i.p.) mesothelioma mouse models. However, whether this is dependent on macrophages in the tumor or in the tumor-draining lymph node (LN) is currently unknown. As DC vaccination depends on tumor-specific T-cell activation in the LNs, we hypothesized that macrophages in the LNs could play a pivotal role in hampering the effectivity of DC vaccination.
Methods: To investigate this, we developed a method whereby LN macrophages are depleted following low-dose intra-pleural administration of clodronate encapsulated liposomes (CEL) 2 days prior to i.p. DC vaccination in mice bearing an i.p. malignant mesothelioma tumor. Via this method, multiple LN macrophage populations were effectively depleted while leaving peripheral and tumor-infiltrating myeloid cells intact. T-cell activation was monitored in peripheral blood 3 days after DC vaccination. Ten days after DC vaccination, mice were sacrificed and tumor weight and intra-tumoral T-cells were characterized.
Results: Depletion of macrophages in the tumor-draining LN prior to DC vaccination improved $CD4^+$ and $CD8^+$ T-cell proliferation in peripheral blood, and drastically reduced tumor weight as compared DC-vaccination alone. Furthermore, mice in which LN macrophage depletion was combined with DC vaccination had increased frequencies of tumor-infiltrating $CD4^+$ T-cells as compared to treatment with DC vaccination alone. In addition, $CD8^+$ T-cell phenotype was markedly improved as evidenced by decreased PD-1 and LAG-3 expression indicating a less-exhausted state.
Conclusion: These results indicate that depletion of LN macrophages, similarly to systemic macrophage depletion, improve anti-tumor immune responses induced by DC vaccination, indicating that LN macrophages crucially hamper DC vaccination effectiveness. Therefore, localized macrophage depletion could offer a new therapeutic strategy for optimizing DC vaccination.

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Trapping TGF- β with decoy receptors restricts TGF-β mediated immune cell suppression

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Cancer progression relies on avoiding immune surveillance and developing an immunosuppressive environment that hinders the anti-tumor immune response. While enhancing immune cell activation has proven to be a promising immunotherapy for some patients, the treatment is still limited by factors that alter immune function in and around the tumor microenvironment. One such immunomodulatory factor is transforming growth factor-β (TGF- β). TGF-β is involved in the regulation of many cellular processes during development and homeostasis of most tissues, and perturbations in TGF-β signaling often lead to tumorigenesis. Elevated production of this multifunctional cytokine has been found in a variety of cancers. In a tumor microenvironment, TGF-β is known to promote growth, invasion, and metastasis of cancer cells, while also aiding in dampening the adaptive immune response. TGF- β allows for cancer immune evasion by restricting the differentiation and activation of anti-tumor T cells.

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To address this challenge, we used phage-display technology to engineer a TGF-β binding protein (termed 6Fc) that has a high affinity for active TGF- β. The reagent identifies active TGF-β on cell surfaces, as shown by flow cytometry, and neutralizes TGF-β biological activities, such as epithelial to mesenchymal cell transition. We hypothesized that if 6Fc can serve as a decoy receptor to ablate TGF-β signaling in T cells, then we would expect T-cells to be resistant to TGF-β mediated suppression. For these purposes, we generated a transmembrane 6Fc fusion protein, and used retroviral transduction to express it in primary antigen-specific T cells. The data show that T cells expressing the 6Fc protein are resistant to TGF- β mediated suppression of activation markers and IFN-γ production. Currently, we are in the process of developing *in vivo* models to evaluate the anti-tumor impact of blocking TGF- β signaling in antigen-specific T cells.

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Pre-clinical validation of a TCR redirected universal NK cell line

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Adoptive transfer of T-cell receptor (TCR)-engineered T cells relies on modifying the patient's own T cells *ex vivo* and re-injecting them. The infusion of T cells in a tailor-made setting involves two costly steps: isolation by leukapheresis and transformation of the cells to redirect them. In some cases the manufacturing time might be longer than the life expectancy of the patient. A way to overcome the preparation of the patient's own T cells is to use ready-made cells. We used the FDA approved Natural killer (NK) cell line, NK-92, and turned it into a T cell by expressing the CD3 signalling complex. This cell line, called Universal Killer (UK)-92 was transfected with therapeutic TCR and shown to mimic redirected killer T cells phenotypically and functionally. Indeed, UK-92-TCR formed what resembled immunological synapses on peptide MHC (pMHC) substrate. In addition, it could be specifically triggered by pMHC to kill a target and release cytokines. Interestingly, UK-92-TCR also adapted their metabolism after TCR clustering. These data clearly suggest that NK cells possess the entire attribute to respond to TCR stimulation. Finally, we show that in a mouse xenograft model, UK-92-TCR could control the tumour growth in a TCR-dependent manner. Taken together, our method provides an innovative means to indefinitely produce TCR-redirected lymphocytes at lower cost and with similar therapeutic value as redirected T cells.

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Inhibition of IL-10 secretion in CAR-engineered NK cells modulates polarization of bystander macrophages and enhances dendritic cell maturation

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Natural killer (NK) cells play a critical role in antitumor immunity by directly eliminating malignant cells and by regulating tumor-specific adaptive immune responses. NK-cell-based cancer immunotherapies typically rely on adoptive transfer of allogeneic NK cells derived from a suitable donor. To enhance

their antitumor activity, NK cells can be further engineered to express chimeric antigen receptors (CARs) that facilitate selective recognition and killing of tumor cells, with early stage clinical development of such approaches ongoing. Nevertheless, in addition to pro-inflammatory cytokines such as interferon (IFN)-γ, activated NK and CAR NK cells can secrete significant levels of immuno-regulatory interleukin (IL)-10, which may counteract pro-inflammatory factors and dampen tumor-suppressive activities of bystander immune cells in the tumor microenvironment.
Using continuously expanding ErbB2 (HER2)-specific CAR NK cells derived from the human NK cell line NK-92 as a clinically relevant model, we aim to better understand the role of NK-cell-derived IL-10 in the interaction of CAR-engineered NK cells with host immune cells within solid tumors. To investigate this, we developed an IL-10-specific endoplasmic reticulum (ER)-retained intracellular antibody (anti-IL-10ER), which upon expression in CAR NK cells trapped the IL-10 protein in the secretory pathway and abrogated IL-10 secretion to an extent comparable to CRISPR/Cas9-mediated IL-10 gene knockout. Importantly, prevention of IL-10 secretion by anti-IL-10ER did not negatively affect proliferation of NK-92 cells, natural and CAR-mediated cytotoxicity, and activation-induced production of IFN-γ. However, downregulation of IL-10 production in activated CAR NK-92 cells enhanced polarization of bystander macrophages towards a tumor-suppressive M1-like phenotype and supported maturation of dendritic cells *in vitro*. These data suggest an enhanced therapeutic potential of IL-10-depleted CAR NK cells, which is currently being analyzed further in immunocompetent mouse tumor models.

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A new cellular immunotherapy for the treatment of multiple myeloma without development of graft-versus-host disease

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Multiple myeloma (MM) is a malignant plasma cell disorder that accounts for approximately 10% of all hematological cancers. Despite recent advances, long-term survival is rare after autologous bone marrow transplantation (auto-BMT) and/or treatment with recently introduced anti-myeloma agents, and disease recurs in virtually all patients. From the other side, allogeneic bone marrow transplantation (allo-BMT) is an effective treatment that can provide partial or complete remission for patients with MM. The therapeutic potential of allo-BMT is attributed to the "graft-versus-myeloma" (GvM) effect that aims to destroy residual tumor cells that survived an induction protocol of chemotherapy/radiotherapy and to maintain immune surveillance to prevent relapse. However, allo-BMT remains a controversial treatment, since the donor T cells that mediate the GvM effect are also the source of the cells that react to other tissue alloantigens and induce graft versus-host disease (GvHD), a major cause of morbidity and mortality in allo-BMT recipients. Nonetheless, allo-BMT remains the only potentially curable treatment for MM. Recent TCR Vβ CDR3-size spectratype analyses in an animal model of MM identified T cells subfamilies involved in the anti-host and anti-tumor reactivity. Based on these results, we tested the potential of integrating auto-BMT with a donor lymphocyte infusion (DLI) composed only of anti-MM reactive Vβ 2, 3 and 8.3 T cell subfamilies. The results demonstrate that these T cell subsets are indeed involved in the generation of a GvM response in MM bearing mice and enhancement of survival. Importantly, this GvM response was not accompanied by the development of GvHD. Nonetheless, the GvM response was not sufficient to completely inhibit relapse. Next, we pre-stimulated donor T cells with MM cells *in vitro* in the presence of co-stimulatory factors and found that, our selective DLI protocol induced a vigorous and long-lasting GvM which translated into long-term survival in the complete absence of GvHD. Interestingly, we obtained almost a similar result by treating MM-beraing mice with repeat doses of naïve donor T cell subfamilies. The treated mice showed lower serum paraprotein levels and lower myeloma infiltration in bone marrow and spleen. Taken together, the results suggests that a transplantation protocol involving only tumor-reactive donor T cell subfamilies can be devised for MM patients that results in

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enhanced survival without symptoms of GvHD.

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Quantitative live-cell imaging as an efficient tool for the assessment of safety and efficacy of engineered T cells and bispecific molecules

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T-cell receptor (TCR)-based immunotherapy has emerged as a promising perspective for cancer treatment over the last years. The ability to kill tumor cells while sparing healthy tissues is the key differentiator of successful TCR-based immunotherapies.
After identification and characterization of high avidity TCRs within our proprietary TCR discovery platform, we are pursuing two approaches to equip primary T cells with the ability to recognize tumor cells. 1) The identified TCR genes are directly used to reprogram primary T cells for ACT or 2) the TCRs are reformatted to single chain TCRs and fused to a T cell-engaging antibody domain to create soluble bispecific molecules.
To monitor tumor cell killing and sparing of healthy tissues by our TCR-modified T cells, we make use of the IncuCyte Zoom system, which enables us to extend our scope beyond traditional endpoint assays such as cytokine release and LDH or ^{51}C -release assays. This quantitative live-cell imaging tool allows high throughput and long-term screening of T cell co-cultures with tumor cells or healthy primary tissues to determine cytolytic activity of our T-cell products. We quantify survival of target cells labeled with fluorescent dyes and additionally visualize cell death using apoptosis markers.
We assessed different cell dyes and a wide array of different tumor cell lines and primary healthy tissues to identify optimal staining conditions for time course experiments lasting at least 72h. Especially primary tissue cells can be sensitive towards expression of fluorescent dyes via viral transduction or even non-viral dyes, which makes a thorough assessment of suitable reagents necessary. We further analyzed the killing of tumor cells over time by bispecific molecule-mediated cytotoxicity as well as direct killing by TCR-transduced T cells, using multiple TCRs recognizing different tumor-related antigens. In addition, the safety profile of various TCRs was assessed in co-culture experiments with primary healthy tissues cells.
In summary, the quantitative Live-Cell Imaging is an efficient tool for the assessment of safety and efficacy of T-cell immunotherapies including TCR-based adoptive cell therapies and T-cell engaging bispecific compounds.

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Preclinical characterization of an off-the-shelf chimeric antigen receptor-engineered NK cell therapeutic for adoptive cancer immunotherapy

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Genetic modification of NK cells with chimeric antigen receptors (CARs) is receiving increasing attention as an approach for adoptive cancer immunotherapy. The clinically applicable cytotoxic cell

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line NK-92 provides an unlimited source of effector cells to investigate and improve CAR concepts for NK cells, and holds potential for development as a standardized off-the-shelf therapeutic. We genetically engineered NK-92 cells to express an ErbB2 (HER2)-specific CAR harboring a composite CD28-CD3ζ signaling domain. GMP-compliant protocols for vector production, lentiviral transduction and expansion of a gene-modified single cell clone (NK-92/5.28.z) were established. Functional analysis of NK-92/5.28.z cells revealed high and stable CAR expression, and selective cytotoxicity against ErbB2-expressing but otherwise NK-resistant tumor cells of different origins. Importantly, upon activation NK-92/5.28.z cells secreted high levels of proinflammatory factors without upregulating immune checkpoint receptors, with CAR-mediated cytotoxicity not being affected by immunosuppressive factors such as a hypoxic environment and high TGF-β concentrations.
To develop these cells for treatment of ErbB2-positive glioblastoma (GBM), we evaluated the activity of NK-92/5.28.z against GBM cell lines and primary GBM cultures, and demonstrated selective ErbB2-dependent cell killing in vitro. Potent in vivo antitumor activity of NK-92/5.28.z was observed in orthotopic GBM xenograft models in NSG mice, leading to a marked extension of symptom-free survival upon repeated stereotactic injection of CAR NK cells into the tumor area. In immunocompetent mice, local therapy with NK-92/5.28.z cells resulted in cures of transplanted syngeneic GBM, induction of endogenous antitumor immunity and long-term protection against tumor rechallenge at distant sites. Of note, irradiation of NK-92/5.28.z cells as a safety measure did not influence in vitro and in vivo antitumor activity. Our results suggest adoptive transfer of ErbB2-specific NK-92/5.28.z cells as a promising new immunotherapy approach for GBM. A phase I clinical trial investigating NK-92/5.28.z cells as a treatment for recurrent ErbB2-positive GBM is ongoing (NCT03383978; clinicaltrials.gov).

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Mouse models of pancreatic cancer for development of combinatorial immunotherapies involving cytostatic agents and agonist anti-CD40 antibody

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Pancreatic ductal adenocarcinoma (PDAC) is the most common malignancy of the pancreas and difficult to treat. 80-90% of patients have disease that is surgically incurable at the time of clinical presentation. In spite of recently improved chemotherapeutic regimens, e.g., involving Nab-Paclitaxel or FOLFIRINOX, the median overall survival time of these patients is a mere 9-11 months.
Immune checkpoint inhibitors, in particular PD-1- and CTLA-4-blocking antibodies, did not show promising outcome in PDA patients, in spite of the presence of tumor-reactive T cells and considerable number of tumor mutanome-encoded neo-antigens (Poschke et al., 2016). The reason for this being the hostile and immunosuppressive tumor microenvironment (TME) composed of a dense fibrotic stroma of extracellular matrix components and a variety of immunosuppressive cells (MDSCs, TAMs, Tregs).
In order to develop effective immune-based therapies and overcome resistance to checkpoint inhibition, we developed preclinical models for PDAC recapitulting the human disease and make use of them to evaluate the efficacy of our immunotherapeutic strategies. In first proof-of-concept (PoC) experiments with transplantable models (MC-38, B16F10, PDAC_30364), we show that the combination of MEK inhibitors and an agonist anti-CD40 antibody results in control of established tumors. Mechanistic studies revealed a modulation of the tumor microenvironment, with respect to macrophage phenotype and T-cell numbers, which was mediated both by the MEK inhibitors as well as the anti-CD40 antibody.

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Role of the Interleukin-33/ ST2L-axis for the CD8-dependent anti-cancer cytotoxicity - Protease- versus receptor-dependent regulation of Interleukin-33 bioactivity

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Novel cancer therapies target the activation of tumor antigen specific cytotoxic T cells in order to improve treatment efficiency. In this context, the roles of Interleukin-33 (IL-33), its membrane-bound signaling receptor ST2L and soluble decoy receptor sST2 remain to be elucidated. The alarmin IL-33 is a member of the IL-1 cytokine family, is released upon necrosis and induces anti- and pro-inflammatory immunity. IL-33 bioactivity is increased by proteases and inhibited by caspases-3 and -7, sST2 and oxidation. Previous work from our group has provided proof of ST2L expression on murine cytotoxic T cells with induction of Th1 immunity driven by IL-33 together with other pro-inflammatory cytokines. These data are consistent with the increasing clinical relevance of IL-33 and sST2 as biomarkers in a variety of diseases, especially in cancer. We hypothesize an essential role of IL-33 in the induction of tumor-antigen specific CD8+ T cell cytotoxicity. Due to the postulated pro-

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inflammatory interplay between CD8+ T cells and IL-33, we aim at identifying local and systemic regulatory mechanisms of IL-33 bioactivity and performed *in vitro* bioactivity assays and affinity measurements. Recombinantly generated IL-33 isoforms aa (amino acid) 95-270 and aa111-270 potently activated ST2L-expressing HEK reporter cells, with the molecule length determining the signal amplitude. In accordance with these results, hyperactive IL-33 aa111-270 exhibited a lower affinity towards sST2 (2.21 nM ± 1.1) than the less bioactive isoform IL-33 aa95-270 (13.47 ± 2.9 nM). However, free IL-33 and IL-1b detected by ELISA in serum samples from healthy male blood donors (n=30) did not induce a corresponding bioactivity, pointing to a systemic inactivation of bioactive IL-33. Incubation of exogenous recombinant IL-33 in human plasma from healthy volunteers (n=3-10) resulted in a significant reduction of bioactivity compared to medium controls. Heat-sensitive proteases and oxidation have further been excluded as elicitors for this effect. Peripheral blood mononuclear cells were shown to express ST2L and sST2 mRNA. Isolated unstimulated CD8+ T cells furthermore expressed ST2L on the cell surface, supporting our hypothesis of IL-33-dependent induction of Th1 immunity on CD8+ T cells. Our experiments demonstrated that IL-33 bioactivity is highly regulated, suggesting a hyper activation by pro-inflammatory proteases and subsequent systemic inactivation. Understanding the regulatory mechanisms as well as the interplay of IL-33 with pro-inflammatory mediators supporting or counteracting anti-cancer Th1 immunity will enable developing pharmacological tools to enforce antigen-specific anti-tumoral therapies.

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Targeted immunotherapy with oncolytic measles virus vaccines
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Oncolytic viruses selectively replicate in cancer cells, leading to tumor lysis and release of tumor antigens in a highly immunostimulatory context. Thus, oncolytic virotherapy represents an *in situ* tumor vaccination approach. With the approval of T-VEC by the FDA and EMA in 2015 as well as many agents currently in Phase I-III trials, oncolytic virotherapy is entering clinical practice. We are mainly focused on oncolytic measles virus (MV) vaccine strains which have evolved into a versatile vector platform allowing for targeted cancer immunotherapy. In our preclinical research we have developed strategies for targeting on the entry and post-entry level, for MV vector-mediated delivery of cytokines, immune checkpoint antibodies, bispecific T cell engagers and tumor antigens. Using this platform, we generate immunovirotherapeutics tailored to the specific tumor contexture, enabling individualized treatment.
Based on preclinical studies, a clinical phase I/IIa trial combining oncolytic measles virus with the anti-PD1 antibody pembrolizumab for the treatment of advanced gastrointestinal tumors will be launched at NCT in Heidelberg this year. Importantly, the trial is accompanied by a translational research program to identify immunological and molecular correlates of therapeutic activity and targets for future development of cancer immunovirotherapy.

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Fc effector function contributes to the activity of human anti-CTLA-4 antibodies
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Anti-CTLA-4 antibodies mediate durable remissions in patients with advanced melanoma, however such responses are limited to a small subset. Despite its potentially depleting isotype, the contribution of antibody-dependent cell-mediated cytotoxicity (ADCC) and role of Fc gamma receptors (FcγRs) in the activity of ipilimumab in vivo remains unclear. With use of a mouse model expressing human FcγRs, we demonstrate that antibodies of isotype equivalent to ipilimumab and tremelimumab mediate intra-tumoural regulatory T (Treg) cell depletion in vivo, increasing the $CD8^+$ to Treg cell ratio and promoting tumour rejection. Critically, this activity only appears relevant in the context of inflamed tumours, explaining the modest response rates observed in the clinical setting. In this context, engineered antibodies with improved FcγR binding profiles drive superior anti-tumour responses and survival in mouse models. In keeping with these observations, in patients with advanced melanoma, the presence of the germline CD16-V158F polymorphism, which confers a higher binding affinity to FcγRs, is associated with response to anti-CTLA-4 therapy in tumours with high mutational or predicted neoantigen burden. Combination of these two metrics appears to better identify long-term responders than considering mutational or predicted neoantigen burden alone.

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High rate of objective anti-tumor response in 9 patients with primary or recurrent glioblastoma after viro-immunotherapy with oncolytic parvovirus H-1 in combination with bevacicumab and PD-1 checkpoint blockade

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Background: Combination therapy is an emerging concept to improve the cinical effects of oncolytic virus based anti-cancer strategies. The oncolytic H-1 parvovirus (H-1PV) induced markers of immune activation in patients with recurrent glioblastoma in a phase I/IIa trial (ParvOryx01). The goal of this investigation was to enhanced H-1PV efficiency by a combination with immune modulators, namely bevacicumab and checkpoint blockade.
Methods: 9 patients with primary (n=2) or recurrent (n=7) glioblastoma were treated with a combination of H-1PV followed by bevacicumab and PD-1 blockade based on a compassionate use (CU) agreement. 7 of the patients were treated by intratumoral and intravenous injection of H-1PV and 2 patients by intravenous injection only. GMP-grade H-1 virus and all medication was provided by Oryx GmbH&Co KG, Baldham, Germany) on a humanitarian basis. Objective tumor response was analyzed by MRI through an independent neuroradiologist using RANO criteria.
Results: MRI showed objective tumor response in 7 of 9 patients (78%): two complete responses (22%), 5 partial remissions (56%) with tumor reduction between 49% up to 94% and 2 progressive diseases (22%). Both patients with progressive disease showed local anti-tumor responses but developed new lesions. The treatment was well tolerated and lead to clinical improvement in all symptomatic patients.
Conclusion: H-1PV based viro-immunotherapy lead to objective tumor responses in 78% of glioblastoma patients even after all but two patients had failed previous therapies. This is a high response rate in this very difficult to treat tumor entity and it supports further systematic clinical

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development of this novel concept for malignant glioma therapy.

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Oncolytic adenoviruses armed with tumor necrosis factor alpha and interleukin-2 enable treatment of solid tumors with T cell therapies

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Currently, T-cell therapy of solid tumors does not provide impressive response rates. Further stimulation of the immune system is necessary and in particular, reduction of immunosuppression is required. We have studied oncolytic adenovirus coding for human tumor necrosis factor alpha (TNFa) and interleukin-2 (IL2) (Ad5/3-E2F-d24-hTNFa-IRES-hIL2, a.k.a. TILT-123) as enhancer of adoptive cell therapies in solid tumors. The studies were conducted in immunocompetent Syrian hamsters with subcutaneous pancreatic tumor model, where the animals were treated with TILT-123 and tumor-infiltrating lymphocytes (TIL). In addition, an immunocompetent mice bearing B16-OVA melanoma were treated with replication-incompetent adenoviruses coding for murine versions of the cytokines (Ad5-CMV-mTNFa and Ad5-CMV-mIL2) and T cells bearing OVA-specific receptors (TCR) or anti-PD-1 antibody. Cytokine-armed adenoviruses were able to enhance the efficacy of each of the combinational therapies (TIL, TCR, and anti-PD-1) curing all the animals receiving the armed virus together with TILs or anti-PD-1. Moreover, the treatment induced system-wide immunological effects seen as protection against tumor re-challenge and as growth control of contralateral untreated tumors. The efficacy was related to increased CD8+ T cell and natural killer cell tumor infiltration, as well as to dendritic cell maturation and diminished presence of immunosuppressive M2 macrophages in tumors. Moreover, TILT-123 improved the safety and efficacy of adoptive cell transfers and allowed leaving out lympho-depleting preconditioning and postconditioning with recombinant systemic IL-2 in both animal models. To conclude, TILT-123 enables curative adoptive cell therapy of solid tumors. The approach will be studied in a clinical trial starting in 2018.

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Intratumoral RNA treatment: a promising approach for the therapy of solid tumors

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Over the last decade new therapeutic strategies to treat cancer by breaking the cancer-induced immune tolerance have garnered considerable interest. The efficacy of immuno-therapeutic strategies is hampered by an immuno-suppressive tumor microenvironment which can be classified in the immune-desert, the immune-excluded and the inflamed phenotype. Each phenotype restrains effective anti-tumoral immune responses by different means, e.g. lack of T-cell priming, activation and/or infiltration, immunosuppressive cytokine milieu or infiltration of immune suppressor cells. Thus, changing the immunosuppressive microenvironment into an immunogenic phenotype in order to e.g. induce efficient intratumoral T-cell priming, enhance T-cell infiltration and functionality and/or induce tumor cell apoptosis to liberate endogenous antigens represents an attractive therapeutic strategy. To reach these goals intratumoral immunotherapy can be applied thereby using the cancer cells as their own vaccines without the need of prior antigen identification or restriction to distinct HLA types. mRNA represents a promising approach for intratumoral immunotherapy as it allows for multifaceted therapeutic interventions by in situ expression of any immunmodulating protein of interest.

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In our study we demonstrated that intratumoral application of mRNA resulted in protein expression as early as 5h post injection in a dose-dependent manner. Evaluation of cytokine/chemokine profile of the injected tumor tissue revealed the transient induction of a proinflammatory tumor microenvironment. Importantly, intratumoral treatment of tumor bearing mice using IL-12 encoding mRNA as relevant target either alone or in combination with non-coding, immunstimulatory RNA substantially reduced tumor growth in tumor-bearing mice with complete remission in up to 50 % of treated mice. Cured mice were protected against a second tumor challenge performed at least 100 days after the first tumor cell inoculation demonstrating the establishment of anti-tumoral memory responses. The anti-tumoral immune response was underlied by increased CD4+ and CD8+ T-cell infiltration in treated tumors compared to non-treated tumors.
In summary, our results demonstrate that intratumoral application of RNA represents a promising therapeutic approach for the local treatment of solid tumors by enabling systemic anti-tumor immune responses.

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Human interleukin-2 (IL-2)/anti-IL-2 antibody complexes preferentially stimulate effector cells and show strong efficacy in tumor models

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Recombinant human interleukin-2 (hIL-2/Proleukin®) was the first immunotherapy agent which demonstrated efficacy in controlled clinical trials and has been approved for the treatment of metastatic melanoma and renal cell carcinoma. However, the therapeutic window of hIL-2 is limited by the short half-life, dose limiting toxicity and expansion of immunosuppressive $CD4^+$ T regulatory (Treg) cells limiting the efficacy of the therapy. We have recently generated and characterized a unique antibody against hIL-2 (called NARA1) blocking specifically the binding of hIL-2 to CD25 (IL-2Ra subunit), thus limiting the expansion of Treg cells while favoring the stimulation of $CD8^+$ T and natural killer (NK) cells [Arenas-Ramirez et. al., Science Translational Medicine, 8, 367ra166 (2016)]. We have now generated a humanized version of NARA1 (hNARA1) with optimal affinity to hIL-2 while conserving the binding epitope. In vivo, the selective expansion of $CD8^+$ T and NK cells is observed when using hIL-2/hNARA1 complexes. The complexes also have a markedly prolonged in vivo half-life relative to hIL-2 and significantly decrease the hIL-2-mediated adverse effects such as pulmonary edema as measured by lung weight. In a transplantable melanoma model, the hIL-2/hNARA1 complexes are highly efficacious in controlling tumor growth. Collectively, our data demonstrate that hIL-2/hNARA1 complexes can overcome the critical limitations associated with hIL-2 therapy and may provide a better therapeutic window for cancer patients. Further development of these complexes toward first-in-man studies is ongoing.

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Abrogation of the immunosuppressive tumor microenvironment in cholangiocarcinoma by targeting PD-1 or GITR

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Background and aims: Cholangiocarcinoma (CCA) is an aggressive hepatobiliary malignancy. The majority of CCA-patients present with advanced disease for which no curative treatment is available. Whether CCA is responsive to immune checkpoint antibody therapy is unknown, and knowledge of the tumor immune micro-environment in CCA tumors is very limited. Therefore, we characterized CCA tumor-infiltrating leukocytes (TIL), determined which co-stimulatory and co-inhibitory molecules are over-expressed on TIL, and assessed the effects of targeting these molecules in ex vivo CCA TIL cultures.
Patients and methods: Tissue sections of resected CCA tumors were immunohistochemically stained for CD4, CD8, Foxp3, and PD-L1 and numbers of positive cells per microscopic field were counted inside the tumors, in adjacent tumor-free liver tissues, and at the tumor margins. Leukocytes were isolated from freshly resected tumors, tumor-free liver (TFL) tissues and peripheral blood of CCA patients, and immune cells were phenotyped by flowcytometry. Effects of soluble GITR ligand (GITRL), or therapeutic antibodies against PD-1 (nivolumab) or CTLA4 (ipilimumab), on T-cell proliferation and cytokine production in *ex vivo* cultures of CFSE-labeled TIL stimulated by CD3 plus CD28 antibodies were determined.
Results: Compared with TFL, proportions of $CD8^+$ CTL, NKT cells and NK cells were decreased, whereas proportions of CD4+Foxp3+ Treg were increased in TIL. Treg were present within tumors, whereas the vast majority of $CD8^+$ CTL and $CD4^+$ Th were sequestered at the tumor margins. Tumor-derived $CD8^+$ T cells showed reduced expression of perforin and granzyme compared to those in TFL and blood. The co-stimulatory receptor GITR as well as co-inhibitory receptors PD-1 and CTLA4 were over-expressed on tumor-infiltrating CD8+ CTL, CD4+Foxp3- Th and CD4+Foxp3+ Treg compared with their counterparts in TFL and blood. PD-L1, CD86 and CD80 were expressed on antigen-presenting cell subsets in tumors, but GITRL not. PD-L1 was also expressed on tumor cells in 10 out of 14 tested CCA patients. Addition of GITRL to *ex vivo* polyclonal TIL stimulations significantly enhanced CD8+ and CD4+ T cell proliferation as well as IFN-γ and TNF-α production, while addition of nivolumab increased CD8+ TIL proliferation and IFN-γ production and addition of ipilimumab only CD8+ TIL proliferation.
Conclusions: Decreased numbers of cytotoxic immune cells with low expression levels of cytotoxic effector molecules and increased numbers of regulatory T cells in tumors, together with over-expression of PD-1 and CTLA-4 on intra-tumoral CTL and Th cells and expression of their ligands in tumors, suggest that the tumor microenvironment in CCA is immunosuppressive. Targeting GITR or PD-1 enhances the functions of CCA tumor-infiltrating T cells ex vivo, indicating that these two immune checkpoints are potential targets for immunotherapy in CCA.

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Investigation of combined immune checkpoint blockade in human malignant pleural mesothelioma

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Till today, human malignant pleural mesothelioma (MPM) remains an aggressive cancer with a poor prognosis due to the limited impact on overall survival of the current treatments. Data from us and others about the presence of the immune checkpoint-related molecules PD-1, PD-L1, TIM-3 and LAG-3 in MPM lay the basis to evaluate their suitability as immunotherapeutic targets. Two clinical trials that investigated PD-1 and PD-L1 inhibition in mesothelioma (KEYNOTE-28, JAVELIN trial) have shown

promising results with room for improvement. It is of great interest to investigate the effect of combined treatments and compare them to stand-alone treatment to select the best therapeutic strategy for MPM.
Human cell lines representative for the epithelioid (NCI-H2818 and NCI-H2795) and sarcomatoid (NCI-H2731) subtypes of MPM were placed in allogeneic co-cultures with healthy donor peripheral blood mononuclear cells. The co-cultures were treated with the following immune checkpoint blocking antibodies: anti PD-1 (Nivolumab®, BMS) or anti PD-L1 (Durvalumab®, AstraZeneca) in combination with anti TIM-3 or anti LAG-3. Supernatant was collected and enzyme-linked immunosorbent assays and multiplex electrochemo-luminescence were used to look at the secretion of 7 cytokines, being IFNg, IL-2/5/6/10, IL-1b and TNF-a, as well as the enzyme granzyme B. Statistical analysis was done to investigate the differences between the treatment conditions.
Treatment with immune checkpoint blockers as monotherapy or in combination resulted in a significant increase in the secretion of granzyme B and the cytokines IFNg, IL-2, IL-5 and IL-10. Although the increased secretion was not always statistically significant for all 3 MPM cell lines of the two subtypes, the same trends were observed among them. Interestingly, highest concentrations of granzyme B and these 4 cytokines were noticed for monotherapy treatment with anti PD-1, anti PD-L1 or either of these antibodies with anti TIM-3. In vivo investigation of PD-1 or PD-L1 blockade in combination with TIM-3 or LAG-3 blockade is currently ongoing to validate our in vitro results.
Our data show that treatment with anti PD-1, anti PD-L1 or their respective combination with anti TIM-3 resulted in the highest secretion of cytokines and granzyme B, suggesting that these treatments stimulate the antitumor response the most. In vivo experiments are currently ongoing for validation.

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Activin-A protects against lung cancer progression by potentiating anti-tumor immunity

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Lung cancer represents the most commonly-diagnosed cancer globally. The immune system constrains cancer development, mostly through the generation of T cell-mediated effective immune responses. Considering the crucial role T cells play in the induction of protective anti-tumor immunity, the identification of factors that can specifically enhance T cell-driven responses in vivo represents a key therapeutic approach for lung cancer eradication. Activin-A is a cytokine that exerts both beneficial and detrimental effects on immune responses depending on the spatiotemporal context. It is overexpressed in patients with lung cancer and is associated with metastasis; still, its role in the development of T cell-mediated anti-tumor responses remains unexplored.
In order to evaluate whether in vivo administration of activin-A confers protection against lung cancer development, we utilized a syngeneic mouse lung cancer model induced by Lewis Lung Carcinoma (LLC) cells and a model of melanoma pulmonary metastases induced by B16F10 cells. Activin-A or PBS was administered intraperitoneally one day following cancer cell transfer and then every three days until sacrifice. Importantly, to decipher the in vivo effects of activin-A specifically on T cell-mediated immune responses in lung cancer we disrupted activin-A's signaling on $CD4^+$ T cells using an inducible model of $CD4^+$ T cell-specific knockout of activin's A type I receptor, ALK4, ($CD4^{ERt2/Cre}$/$Acvr1b^{fl/fl}$). These mice were subsequently inoculated with LLC cells to induce primary lung tumors.
Our findings demonstrate a significant increase in activin-A serum levels in lung tumor bearing mice, accompanied by enhanced expression by bronchial epithelial cells at the tumor site. Notably, therapeutic administration of activin-A in distinct mouse models of lung metastasis, induced a marked regression in cancer progression, evidenced by macroscopic, PET/CT imaging and histological studies, concomitant with a greatly extended overall survival. Activin-A anti-tumor effects were associated with increased infiltration of IFN-γ, TNF-α and IL-17 secreting $CD4^+$ T effector cells in lung

tumors and decreased frequencies of Foxp3$^+$ Treg and myeloid derived suppressor cells. Moreover, activin-A treatment significantly decreased the expression of the immune checkpoint inhibitors, PD-1, CTLA-4, Lag3 and Tim3, among lung TILs. Notably, mechanistic studies demonstrated that CD4 T cells were essential in mediating the anti-cancer effects of endogenous activin-A, as disruption of its signaling in these cells, resulted in enhanced tumor progression. This was accompanied by diminished effector responses concomitantly with heightened percentages of Foxp3$^+$ Treg cells and several immune checkpoint inhibitors.
Collectively, our studies reveal a novel role for activin-A in enhancing anti-tumor T cell-mediated responses that may be beneficial for the combat of lung cancer progression.

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Rapid generation of peptide-HLA complexes with disulfide-stabilized empty HLA-A*02 molecules for high throughput screening approaches

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Presentation of peptides on the cell surface by Human Leukocyte Antigen (HLA) molecules is a fundamental component of the immune response against viral infection or cancer. Soluble peptide-HLA complexes are consequently used in many applications ranging from detection or stimulation of peptide-HLA specific cells to receptor-ligand kinetic screenings with its cognate receptor, the T cell receptor (TCR). We here present the development of an alpha-1 to alpha-2 disulfide-stabilized HLA-A*02 molecule that can be readily produced in an empty form and rapidly loaded with peptide without interfering with peptide-HLA:TCR complex formation. The molecule stably binds peptides from a wide range of predicted affinities for the peptide-HLA complex. Affinities of the interaction between TCR and peptide-HLA measured by bio-layer interferometry (BLI) are similar for high and low affinity constants when compared to wild type HLA-A*02 molecules presenting the same peptides. The disulfide-stabilized molecules can be used as immobilized ligands as well as soluble analytes and show high stability, enabling a wide range of use cases in high throughput efficacy or safety screenings. We therefore propose the disulfide-stabilized HLA-A*02 molecule as a novel tool to enable a new level of understanding of the peptide-HLA:TCR interaction and aide in the development of effective and safe cancer immunotherapies.

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Type I IFN responsiveness of melanoma is cell state dependent and can be both harnessed and suppressed to enhance oncolytic virotherapy efficac

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Oncolytic virotherapy is a new promising approach to treat malignant melanoma. Tumor cells are often permissive for viral infection and oncolysis due to their active metabolism and decreased responsiveness to type I interferons (IFN-I). However, also IFN-I responsive tumors may be suitable targets for oncolytic virotherapy, as local IFN-I responses have often been associated with anti-tumor immunity. The underlying mechanisms accounting for the differences in IFN-I responsiveness of melanoma are poorly understood. The aim of this work was to analyze the responsiveness of a collection of human and mouse melanoma cell lines to IFN-I utilizing an oncolytic Semliki Forest virus expressing EGFP (SFV-VA7-EGFP). 16 human melanoma cell lines with a spectrum of phenotypes ranging from very melanocytic ($MITF^{high}$) to poorly differentiated ($MITF^{low}$) were screened for their IFN-I responsiveness by treatment with varying concentrations of IFN-I followed by infection. The infection kinetics were monitored with fluorescence and bright field microscopy over 72h, after which the net result of cell proliferation was quantified using crystal violet staining. Following IFN-I pretreatment, healthy primary melanocytes were readily protected from infection, whereas all melanoma cell lines had, to varying degree, lowered antiviral type I IFN responsiveness. Melanoma cell lines, which had retained partial responsiveness to IFN-I displayed a basal IFN-I signature in a bioinformatic analysis. Interestingly, the one quarter of the melanoma cell lines with poorest IFN-I responsiveness were all melanocytic ($MITF^{high}$), suggesting a potential link between the differentiation status and the responsiveness to type I IFNs. Supporting the hypothesis, MITF overexpression utilizing a tet-ON system in $MITF^{low}$ Mamel65 human melanoma cell line completely abrogated their type I IFN responsiveness allowing productive SFV-VA7-EGFP infection and oncolysis. To test the hypothesis that both suppressing and harnessing the type I IFN responses may be utilized to benefit oncolytic virotherapy, we treated HCmel12 mouse melanomas with SFV-VA7-EGFP in combination with antibodies targeting either the type I IFN receptor or an immunosuppressive PD1 receptor on T cells. While both approaches were found to enhance oncolytic virotherapy efficacy and type I IFN-receptor deficient melanomas were eradicated in Rag-knock out mice, local IFN-I signaling blockade in the tumors before virus administration resulted in marked toxicities suggesting peripheral spread of the infection.

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Immunomodulatory properties of the glyco-engineered anti-EGFR antibody Tomuzotuximab

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Targeting the EGF receptor (EGFR) with monoclonal antibodies is clinical effective. Besides EGFR signaling inhibition, the anti-EGFR IgG1 monoclonal antibody cetuximab shows Fc-mediated immunomodulatory effects on FcγRIIIa expressing immune cells e.g. by stimulating antibody-dependent cell-mediated cytotoxicity (ADCC). However, there is clinical evidence that cetuximab also modulates the phenotype and activation of myeloid cells leading to an altered immune crosstalk and T cell activation. Additionally, cetuximab treated patients show alterations of the tumor microenvironment by regulation of checkpoint molecules on tumor and immune cells. Tomuzotuximab, a glyco-engineered version of cetuximab with optimized Fc glycosylation, allowing for enhanced FcγRIIIa binding, was generated to boost Fc-mediated effector functions. Hence, we hypothesize that glyco-optimized tomuzotuximab is superior in immune activation and regulation of checkpoint molecules on various immune cells.
Glyco-engineering of tomuzotuximab did not change Fab-mediated characteristics as shown for antigen-binding, specificity, affinity, apoptosis induction and inhibition of EGFR phosphorylation as well as tumor cell proliferation inhibition. As expected, reducing the core-fucosylation in the Fc part results in enhanced FcγRIIIa affinity, which improved the expression of the co-activating receptor CD137 on NK cells when co-cultured with EGFR positive tumor cells. Contrary, enhanced cytokine production (e.g. IFNγ) by tomuzotuximab-activated NK cells also leads to stronger upregulation of PDL1 on tumor and immune cells. Monocytes and dendritic cells (DCs) represent further FcγR-bearing and cytokine responsive immune cells. Using co-cultures of PBMCs with tumor cells, tomuzotuximab-stimulated

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monocytes and DCs demonstrate an improved maturation and activation phenotype and enhanced expression of activating (e.g. CD40) and inhibitory (e.g. PD-L1) checkpoint molecules as measured by flow cytometry. In functional assays, tomuzotuximab pre-treated monocytes showed an increased capability to enhance T cell activation. Furthermore, co-cultivation of tomuzotuximab pre-matured DCs with allogeneic T cells in a mixed-lymphocyte reaction results in improved T cell activation but also increased expression of PD-1. Especially, increased PD-1 and PD-L1 levels show promising rationales for future combinatory strategies.
The present study shows that tomuzotuximab is not only superior to increase effector functions of NK cells but also increases myeloid cell maturation resulting in enhanced immune cross-talk and T cell activation. Enhanced regulation of checkpoint molecules shows that tomuzotuximab could be a preferable partner for combinatory immunotherapies. Currently, tomuzotuximab is investigated in a phase 1 clinical study and in depth flow cytometry analysis is used to confirm our *in vitro* findings.

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dermaject® intradermal injection device ensures superficial and reproducible intradermal injections in human *ex vivo* skin

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Background: Intradermal drug delivery, compared to subcutaneous drug delivery, provides numerous benefits, such as significantly more effective vaccine and immunotherapy action, faster bloodstream absorption, and higher bioavailability.
dermaject® intradermal injection device is a novel, CE marked class IIa sterile single use medical device with 30G micro cannula for injection of liquid drugs into the top layer of the skin (intradermal / intracutaneous route) in a depth of 0.77-0.8 mm for drug targeting of the skin, immune and lymphatic systems.
Standard of care for intradermal injections is the Mantoux injection method where injections are performed manually with customary syringes and cannulas. The results depend on the experience of the operator, thus leading to a varying and non-standardized outcome and to an operator specific error rate.
Purpose: The purpose of this study was to demonstrate strictly intradermal injection depots and a high degree of standardization using the **dermaject® intradermal injection device** for injections in human skin.
Methods: Intradermal injections in *ex vivo* human skin were performed using **dermaject® intradermal injection device**, Soluvia device (Becton Dickinson) and the manual method (Mantoux injection technique). Alcian blue solution was injected, histological sections of wheal centers and edges were obtained and hematoxylin and eosin (HE) staining was used to determine dimensions and locations of injected volumes. Depths and distances of infiltrates to the skin surface were measured; mean values and variances were compared using one-way variance, t-test, Levene-test, and f-test analyses.
Results: Injections with 0.1 and 0.2 mL with **dermaject®** resulted in 0.317 mm and 0.324 mm mean distance to the skin surface, compared to 0.778 mm with Soluvia. The injection of 0.1 mL with **dermaject®** had a significantly lower depth compared to Soluvia and the manual method (Mantoux). The variances of the distance to the skin surface were 0.00064 and 0.00053 using **dermaject®** with injection volumes of 0.1 and 0.2 mL, respectively. The variance of the depth with **dermaject®** injections of 0.1 mL was 0.04147. This was significantly lower compared to all other tested injection techniques.
Conclusion: Injection depots with **dermaject®** were located more superficial in the skin compared to the Soluvia device, thus leading to a higher probability of strictly intradermal injections. Injection

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depots with **dermaject®** (0.1 and 0.2 mL, respectively) showed the lowest variances of the distance to the skin surface, compared to Soluvia and the Mantoux method, thus demonstrating better reproducibility and standardization for intradermal injection depth.
Therefore **dermaject®** is a highly relevant option for all parenteral substances and fluids suitable for administration via the intradermal route.

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Loss of myeloid cell GCN2 drives progression in experimental melanomas during immune checkpoint inhibition

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Tryptophan metabolism is a central pathway in immune regulation and is known to shape the immunosuppressive tumor microenvironment. During the past decade inhibitors targeting the rate-limiting enzymes that mediate tryptophan depletion, namely indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), have entered clinical trials. In T cells, the stress kinase general control non-derepressible 2 (GCN2) has been identified as a molecular sensor of tryptophan deprivation that induces apoptosis and attenuates T cell proliferation upon activation.
We have recently demonstrated that T cell GCN2 is dispensable for suppression of T cell-mediated melanoma rejection and revealed that, despite high turnover rates, intratumoral tryptophan concentrations do not drop significantly. Within this current study we investigated whether myeloid cell GCN2 is a critical regulator of anti-tumor immune responses using the experimental B16 melanoma model. Indeed, mice harbouring *Gcn2*-deficient immune cells failed to respond to a combinatorial immunotherapy that consisted of a glycoprotein 100 (gp100) peptide vaccine and immune checkpoint inhibition targeting programmed death-ligand 1 (PD-L1). *In vitro* genetic ablation of *Gcn2* reinforced immunosuppressive capacities of bone-marrow derived macrophages. In addition, GCN2 expression in bone marrow-derived dendritic cells augmented gp100-specific cytotoxic T cell responses.
In conclusion, our results suggest that myeloid cell GCN2, despite stable intratumoral tryptophan levels, is a critical factor that drives anti-melanoma immune responses during checkpoint blockade.

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Dissecting the synergistic effect of chemotherapy and immunotherapy on anti-tumoral T cell functions in breast cancer

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Immunotherapies that target immune checkpoint molecules proved effective for the treatment of several solid tumors. However, the majority of breast cancer patients does not respond to this kind of approach, highlighting the need of new strategies able to elicit an effective anti-tumoral immune response. The combination of conventional and immuno-based therapies represents a promising strategy to elicit such response. The aim of this project is to evaluate the efficacy of immunotherapy

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and chemotherapy combination in primary and metastatic breast cancer, to dissect its effect on the endogenous anti-tumoral cytotoxic CD8+ T cells, and finally to assess the impact of a spontaneously developing tumor on T-cell biology.
For our experiments we used *K14cre*;*Cdh1*$^{F/F}$;*Trp53*$^{F/F}$ (KEP) mice, a spontaneous breast tumor model that closely resembles human invasive lobular carcinoma. Mammary tumors in KEP mice show an altered T-cell balance compared to tumor-free mammary glands, with a decreased infiltration of cytotoxic CD8$^+$ T cells and conventional CD4$^+$ T cells, and increased percentage of T regulatory cells. CD8$^+$ T cells that infiltrate mammary tumors have an increased expression of multiple inhibitory receptors, and decreased IFNg production, compared to peripheral CD8+ T cells of KEP mice. In vitro exposure of facs sorted tumor-derived CD8+ T cells to IL-15 or IL-2 augments their capacity to produce IFNg, indicating that their suppressed state is reversible. Treatment of tumor-bearing KEP mice with anti-CTLA-4 and anti-PD-1 antibodies does not affect the tumor growth, but a synergistic therapeutic benefit is observed when a-CTLA-4 and a-PD-1 are combined with cisplatin, and not docetaxel, in a CD8$^+$ T-cell dependent mechanism.
All together, our data indicates that tumor-infiltrating CD8+ T have impaired functionality but are not terminally dysfunctional. Nontheless, only the combination with chemotherapy and immunotherapy is able to elicit an efficient CD8+ T-cell response, in a drug-dependent manner.
We are currently dissecting the mechanisms underlying the synergistic therapy response, in order to ultimately contribute to the rational design of new immunomodulatory treatment strategies for breast cancer.

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An improved preclinical immuno-oncology platform: Patient-derived tumor xenografts in humanized NSG-SGM3 mice

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The JAX® Onco-Hu® platform combines immune-system humanized mice with engrafted tumors to study the interactions between the human immune system and human cancer in a preclinical *in vivo* setting. Previously, we have shown that humanized NOD-*scid* IL2Rγ^{null} (NSG™) mice engrafted with patient-derived xenografts (PDX) allow to assess the efficacy of check-point blockade. One major goal to improve the system is to generate murine humanized models containing a more complete human hematopoietic system and robust innate immune cell population. Triple-transgenic NSG mice expressing myelosupportive human cytokines KITLG, CSF2, and IL-3 (NSG-SGM3) produce higher myeloid and Treg populations in the circulation when compared to NSG mice when engrafted with CD34$^+$ human hematopoietic progenitor cells (HPCs) from CD3-depleted umbilical cord blood over 18 weeks post engraftment. A range of PDX tumors was implanted into humanized NSG-SGM3 mice 2-3 months post engraftment. The general immune cell infiltrates and specifically the levels of hCD33+ myeloid cells in tumors were analyzed upon tumor dissociation and multicolor flow cytometry. hCD45+ cell infiltration was significantly increased in hu-NSG-SGM3 in the PS4050 melanoma PDX model mice when compared to hu-NSG mice engrafted with the same HPC donor (3.7% vs. 1% of viable cells). The majority of tumor infiltrating cells in hu-NSG-SGM3 mice expressed hCD33 (55% of hCD45+) and the percentage was significantly higher than that in hu-NSG mice (13%). The hCD3+T cell infiltration was similar between the two strains (~20% of hCD45+). When treated with the anti-PD-1 antibody pembrolizumab (Keytruda), PS4050-bearing hu-NSG-SGM3 mice showed a significant reduction in tumor growth and greatly reduced PD-1 levels in tumor infiltrating T cells by flow cytometry analysis. Overall hCD45+ infiltration and the frequencies of hCD4+T, hCD8+T, and hCD33+myeloid cells in tumors remained similar upon treatment. We proved that the effect of Keytruda on tumor growth reduction in hu-NSG-SGM3 mice is PD-L1-dependent by using the human

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lung carcinoma cell line NCI-H460 in which PDL-1 was deleted by CRISPR. PD-L1 KO NCI-H460 cells grew more slowly than mock transfected cells in the presence of Keytruda. Together, these results indicate that PDX tumor-can implanted hu-NSG-SGM3 mice can serve as a tool to study the interactions between the human immune system and the tumor micro-environment a platform for preclinical immuno-oncology efficacy studies.

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The AA protein-based model explains how cancer could arise
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Background: Cancer is a complex disease which continues to defy us besides the colossal efforts and financial means used for the search of its cure. The continuous trend of cancer resistance to a large variety of cancer drugs calls for a paradigm shift. In order to get to root of the problem, current concepts on cancer initiation have been reviewed. Therefore and after having challenged the role of DNA mutations in cancer initiation; the question asked next is: If DNA mutations are not the initiating events in cancer, how then to explain malignancy? A thorough analysis of cancer hallmarks provides us with initial answers.
Results: (*i*) Cancer could result from a major cellular event switching a cell from normalcy-to-malignancy; (*ii*) DNA mutations occur as secondary events following this switch; (*iii*) cancer cells earn a powerful adaptive property which is behind the resistance trend so far observed with all types of cancer therapies; (*iv*)A protein-based model which most likely explains cancer genesis will be presented.
Conclusions: This unprecedented work puts the finger on the core problem of cancer and explains how it could start and develop. This analysis while demystifying cancer; opens the doors on to how to control this disease and lead to its eradication.

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Peptide-based immune system engagers targeting α3 integrin prevent tumor growth and metastasis in a syngeneic triple-negative 4T1 tumor model
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Triple negative breast cancer (TNBC) subtype is classified as one of the most aggressive types of breast cancer and correlates with poor prognosis for the patients despite their chemosensitivity. Due to the lack of hormone receptors estrogen (ER) and progesterone (PR) as well as the human epidermal growth factor receptor type 2 (HER2) targeted therapy using antibodies against these receptors are not effective. The goal of the current study is to develop a unique immunotherapeutic approach using synthetic immune system engagers (ISErs), to be therapeutically applied in TNBC. A peptide-based antibody-like structure (Y9) was generated composed of binding peptides targeting α3 integrin on tumor and stromal cells and an effector peptide activating formyl peptide receptors to engage the innate immune system to induce an anti-tumor immune response. In vitro binding of Y9 to cell lines was tested by flow cytometry measurements. Stimulation of human or murine immune cells was evaluated in a flow cytometry based assay for phagocyte NADPH oxidase. In addition, cytotoxicity was tested by an MTT assay after incubating the cell lines with Y9. *In vivo* efficacy of Y9 was tested in a syngeneic TNBC mouse model using female immune-competent Balb/c mice and the 4T1-luc-GFP cells. Tumor cells were subcutaneously injected in the mammary fat pad together with Y9 or diluent only as control. Tumor growth was monitored by caliper measurements twice per week and metastasis by bioluminescence (IVIS) measurement once a week for 4 weeks. Tumor and organs were

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subsequently analyzed in histopathology and immunohistochemistry.
Binding assays showed binding of Y9 to murine 4T1 breast cancer cell lines with an apparent kD of 25 nM similar to other human cancer cell lines. Activation of NADPH oxidase in human neutrophils occurred at Y9 concentrations of 100-1000 nM, whereas in murine leukocytes 1-10 µM was required to see this effect. Leukocytes of NSG-SGM3 mice engrafted with human CD34+ progenitor cells required the same high Y9 concentrations to be activated. A direct effect of Y9 on 4T1 or MDA-MB-231 cell viability after overnight incubation with up to 1 mM Y9 could not be demonstrated. Orthotopic application of a single dose of 800 nmol of Y9 along with 4T1-luc-GFP cells completely prevented tumor growth *in vivo* after 4 weeks of inoculation. In contrast, control treated animals showed a tumor mass in mammary fat pad as well as metastasis primarily in lung but also in lymph nodes, liver, spleen and kidney when analyzed IVIS measurements and in H&E staining.
In conclusion, we found a strong anti-tumor effect of Y9 in preventing the growth of 4T1-luc-GFP tumors in vivo, however, therapeutic efficacy has to be tested in further experiments.

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Discovery and validation of highly specific T-cell receptors for Immatics's immunotherapeutic applications

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T-cell receptor (TCR)-based immunotherapy has emerged as a promising perspective for cancer treatment over the last years. Therefore, the combination of highly-specific tumor targets and target-restricted TCRs is crucial for success of TCR-based immunotherapies.
Immatics's proprietary target discovery engine XPRESIDENT® offers a unique insight into the tumor immunopeptidome based on differential presentation and expression analyses of more than 600 tumor samples. Immatics uses this wealth of information to identify novel high-priority TUMor-Associated Peptide (TUMAP) targets. These TUMAPs build the foundation for a powerful flow cytometry based TCR discovery platform which encompasses identification and characterization of TUMAP-specific TCRs.
Here we present our advanced approach to identify and characterize TCRs restricted towards selected TUMAP targets using our TCR discovery platform. TUMAP-specific T cells are expanded with the help of artificial antigen-presenting cells prior identification and we established a complementary way based on the identification in a highly sensitive manner directly from the natural T cell repertoire of healthy donors. Subsequent a highly sensitive single cell 5'-RACE approach allows for rapid and efficient TCR sequence identification. This approach enables the collection of large numbers of unique TCR sequences specific for diverse TUMAP targets. After identification and transfer of TCR sequences into expressible formats, multiple validation techniques are employed for high throughput side-by-side TCR characterization to find the most promising candidates. Validation methods range from high-throughput flow cytometry screening for pMHC multimer binding capability to functional avidity measurements, specificity screening with similar peptides, determining the individual TCR binding motif and TCR-pMHC binding affinity determination using soluble TCRs. In addition, we show recognition of endogenously expressed and presented TUMAP targets on tumor cell lines in cytokine secretion tests and image-based killing experiments. Next, we use our in-house on- and off-target specificity and safety characterization process for in-depth characterization of promising TCR candidates. Based on the individual TCR binding-motif, further potentially cross-reactive peptides are identified from our large XPRESIDENT® immunopeptidome database and tested. In addition, TCR candidates are tested for absence of TCR-mediated recognition against a panel of primary cell types from healthy tissues, covering all critical organs and a wide variety of different cell types.
In summary, the combination of comprehensively characterized and validated TCRs reactive against

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unique XPRESIDENT® TUMAP targets equips Immatics with an excellent basis for development of TCR-based immunotherapies including TCR-based adoptive cell therapies and T-cell engaging bispecific compounds.

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Identification and profiling of PX-D26116, a potent Hydroxyamidine-based IDO1 inhibitor
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Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme-containing enzyme catalyzing the rate-limiting step in the kynurenine pathway namely, metabolism of L-tryptophan to N-formylkynurenine. The metabolites from an activated kynurenine pathway as well as local depletion of L-tryptophan can both result in impaired T cell activity. Since tumors often overexpress IDO1, it poses an important mechanism by which many tumors are able to escape immune surveillance.
On this rationale, small molecule inhibitors blocking the enzymatic activity of IDO1 have been developed. These have enabled restoration of immune control and increase response of tumors towards immunotherapy.
Currently, Epacadostat is the most advanced IDO1 inhibitor in clinical development. It is used in a pivotal phase 3 study for the treatment of advanced melanoma in combination with the PD-1 checkpoint inhibitor Pembrolizumab (Keytruda©). Furthermore, Epacadostat is being tested in over 40 clinical studies in various tumor entities with other co-treatments. The available clinical data demonstrate improved response rates when using the Epacadostat/ PD-1 checkpoint inhibitor combination compared to anti-PD-1 monotherapy. Additionally, this combination demonstrates a comparable efficacy to the PD-1/CTLA-4 checkpoint blockade combination, while offering a much more benign safety profile.
However, while being clinically efficacious and safe, Epacadostat is heavily metabolized in humans. In steady state, 8-10 times more inactive metabolites than active drug substance are measured in patients after dosing twice daily. This may limit the therapeutic window as well as the overall efficacy of the drug in cancer patients.
Using the Hydroxyamidine pharmacophore of Epacadostat, we identified a chemical series within a distinct IP space. Both our lead compound PX-D26116 as well as Epacadostat have cellular IC50s in the low nanomolar range, whereas PX-D26116 is superior to Epacadostat in the biochemical inhibition assay. In contrast to Epacadostat, which is readily inactivated by human Phase II metabolism, PX-D26116 exhibits an improved metabolic stability. In vitro and in vivo data will be presented benchmarking our candidate molecule PX-D26116 directly against Epacadostat.
With PX-D26116 we have identified a candidate for further preclinical and clinical development i.e. with an improved metabolic stability vs Epacadostat, which ultimately may translate into a superior clinical profile.

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Nur77, a new target to boost DC vaccination?
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Dendritic cells are the professional antigen presenting cells of the immune system. Proper function of dendritic cells is crucial in eliciting an effective immune response against pathogens and in inducing anti-tumor immunity. Different members of the nuclear receptor family of transcription factors have been reported to affect proper function of immune cells. Nur77 is a member of the NR4A subfamily of orphan nuclear receptors and has been reported to be expressed and function within the immune system. We now show that Nur77 is expressed in different murine dendritic cell subsets *in vitro* and *ex vivo*, in human monocyte derived DCs and in freshly isolated human BDCA1+ dendritic cells, but is expression is dispensable for dendritic cell development in the spleen and lymph nodes. We show, by siRNA-mediated knockdown of Nur77 in human monocyte derived DCs and by using Nur77-/- murine dendritic cells, that Nur77-deficient dendritic cells have enhanced inflammatory responses leading to increased T cell proliferation. Pretreatment of human monocyte derived DCs with 6-mercaptopurine, an activator of Nur77, leads to diminished dendritic cell activation and subsequent T cell activation. Altogether our data show an important role for Nur77 in modifying the activation status of human and murine dendritic cells. Ultimately, targeting Nur77 may prove to be efficacious in boosting dendritic cell function and may lead to the development of improved dendritic cell-based immunotherapies.

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Zoledronic Acid suppresses tumour associated macrophages and myeloid derived suppressor cells in murine HCC

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Background and Objectives: HCC is associated with chronic inflammation leading to recruitment of bone marrow derived cells, mainly tumour associated macrophages (TAM, M2) and myeloid-cell derived suppressor cells (MDSC). Both cell types markedly suppress anti-HCC immune responses and are present in all stages of HCC. Zoledronic acid (ZA) is used in patients with osteoporosis to inhibit osteoclasts, i.e., bone macrophages with TAM-like properties. We hypothesized that ZA can polarize tumour promoting TAM and MDSC towards M1 macrophages with anti-tumour activity.
Methods: Mouse bone marrow derived in vitro M1 or M2 polarized macrophages () were exposed to increasing concentrations of ZA. Transcript levels of M1 markers (TNFα, IL-1β, iNOS, TIMP1) and M2 markers (Fizz1, ARG1, VEGF, YM1, MRC1, MMP-9) were determined by qPCR. IL-12 and SOCS3 (M1), IL-10 and VEGF (M2) secretion were measured by ELISA. Mice bearing a syngenic HCC (DEN/Mdr2KO model) received 3 doses of 100µg ZA/kg BW or vehicle control intraperitoneally weekly for a period of one month from age month 5-6. After four weeks of treatment mice were sacrificed to measure hepatic tumor number and volume and livers were assessed by H&E histology, IHC and qPCR.
Results: ZA dose-dependently decreased M2 (Fizz1, ARG1, STAT6, YM1, MRC1, MMP-9) and increased M1 macrophage (iNOS, TNF-alpha, IL-1beta, SOCS3) specific transcript levels without affecting cell viability in vitro. This was paralleled by a significant upregulation of IL-12 and dowregulation of IL-10 protein secretion into the cell supernatant. Compared to vehicle controls, treatment of DEN/Mdr2KO mice with ZA reduced tumour number and volume by 33.3% and 68%, respectively ($p < 0.004$). Tumour cell proliferation as evaluated via Ki-67-positive cells was significantly blunted by ZA. Staining of YM-1 and glypican positive M2 and CD68 positive total macrophages of ZA treated HCC liver sections showed a significant upregulation of M1>M2 macrophages and reduced numbers of TAM. ZA significantly downregulated hepatic transcript levels the TAM/MDSC markers CSF-1, VEGF, TGFβ1, Cox2, Hif1α, CCL2, CCL3, CCL5 and CCL17.
Conclusions:
1. ZA exhibits potent macrophage (and MDSC) repolarizing activity (M2 towards M1) in- vitro and in-vivo, thereby increasing anti-HCC immune responses and limiting angiogenesis.
2. Since ZA has a well-known clinical safety profile, it should be assessed alone or in combination with

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other anti-cancer agents in patients with HCC.

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HPN424, a half-life extended, PSMA/CD3-specific TriTAC for the treatment of metastatic prostate cancer

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New therapies are urgently needed to treat metastatic, castrate-resistant prostate cancer (mCRPC). In the European Union, roughly 71,000 patients die from mCRPC each year. Once metastasized beyond regional lymph nodes, the 5-year survival rate is 30%. While novel therapeutics like abiraterone and enzalutamide have improved the treatment options for mCRPC, no curative treatment is available.
HPN424 is an antibody derivative called TriTAC (Tri-specific T cell Activating Construct) under development for the treatment of mCRPC. The TriTAC platform addresses shortcomings of existing T cell engagers, including short serum half-life, limited tissue penetration, and suboptimal activity.
HPN424 is designed to simultaneously bind to CD3ε on T cells and to prostate specific membrane antigen (PSMA, FOLH1) on prostate cancer cells. PSMA is expressed in >90% of malignant lesions of patients, and outside the central nervous system, its expression on normal tissue is largely restricted to the prostate. A third domain of HPN424 binds non-covalently to serum albumin for extension of serum half-life. HPN424 has a molecular mass of approximately 50 kDa, i.e., one third of the size of a monoclonal antibody. This smaller size is expected to allow for faster diffusion of the TriTAC into human tumor tissues than is possible with regular antibodies given the high interstitial pressure and dense extracellular matrix in solid tumors.
HPN424 efficiently binds PSMA on human prostate cancer cell lines with an affinity of 0.5 nM. When incubated in co-cultures with resting human T cells and prostate cancer cells, the TriTAC activates T cells leading to cytokine production, T cell proliferation, and redirected target cell killing with EC_{50} values in the single digit picomolar range. When administered to mice bearing human prostate cancer xenografts and human T cells, HPN424 eradicates subcutaneous tumors.
In studies with non-human primates, HPN424 was well tolerated. The affinities of HPN424 for human and cynomolgus monkey CD3 and albumin are comparable, while HPN424 binds only marginally to cynomolgus PSMA and therefore, target-mediated findings were limited. Dose-dependent, transient CD3-mediated pharmacodynamic changes involving cytokines and T cell activation markers were observed. Pharmacokinetic analysis and serum half-life supports weekly administration of HPN424 in humans.
Our preclinical data suggest that HPN424 is highly efficacious and safe and can be conveniently administered to treat patients with mCRPC.

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Identification of exceptional therapeutic anti-CD40 agonists by optimal exploitation of rabbit antibody diversity through single B-cell cloning

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The TNF-receptor family member CD40 is an important costimulatory receptor on antigen presenting cells and the therapeutic activation by agonistic antibodies or the soluble ligand has been a major

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focus of preclinical and clinical R&D for the past years. Therapeutic antibodies in development, such as CP-870,893 (RG7876), have shaped the current perception on the functional requirements of agonistic CD40 antibodies implying that cross-linking of CD40-bound antibodies by accessory cells through Fc-receptor binding (in particular FcγRIIb) is a prerequisite for eliciting therapeutically meaningful activation of CD40 signaling. At the same time, therapy with CP-870,893 is associated with serious adverse events and an MTD as low as 0,2 mg/kg.
MAB Discovery successfully applies a unique technical and conceptual approach to functional diversity of antibodies based on B-cell cloning from immunized wild-type rabbits. Using our technology, we sought to generate a portfolio of agonistic CD40 targeting antibodies, with the highest possible diversity of paratopes (binding domains). In addition, we chose to use an Fc-receptor silent IgG1 modality to identify potent agonists which act independent of Fc-mediated cross-linking. The results are remarkable. We have generated and tested more than 300 humanized, Fc-receptor silent, agonistic CD40 antibodies, which potently activated NF-κB signaling in a cell-based reporter gene assay. Subsequently, we applied a monocyte-derived dendritic cell maturation assay in which the Fc-receptor binding-dependency of CP-870,893 can be clearly demonstrated. Strikingly, among the Fc-receptor silent antibodies tested in this assay, we identified a fraction with outstanding agonistic activities, exceeding even those of Fc-optimized variants of CP-870,893.
Therefore, the results of this CD40 antibody generation campaign demonstrate that potent, Fc-independent activation of TNF receptor family targets can be achieved and that striving for a maximal functional diversity of antibodies with different paratopes is a very successful strategy to find unique antibodies. Agonistic antibodies of this kind will provide a better risk to benefit ratio for patients and a controllable pharmacology.

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Identification of a naturally presented Myeloma-associated BCMA peptide as an immunogenic T-cell epitope for immunotherapeutic approaches

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Immunotherapeutic strategies such as monoclonal antibodies, adoptive T-cell therapy, or CAR T cells, are demonstrating a positive impact on survival by targeting various MM-associated antigens. One feasible target antigen for immunotherapy is represented by the B-cell maturation antigen (BCMA). BCMA is selectively expressed by cells of the B-lineage including multiple myeloma cells. Hence, BCMA has been evaluated as a promising target for immunotherapeutic approaches such as CAR T cells or monoclonal antibodies showing positive effects in phase I clinical trials. A further attractive approach might also be the use of cytotoxic T cells bearing T-cell receptors recognizing BCMA-derived peptides. Therefore, the identification and characterization of BCMA-derived peptides, which are naturally presented by human leukocyte antigens (HLA) and thus serving as target structures for $CD8^+$ T cells (with or without a genetically modified T-cell receptor) is indispensable.
In a previously conducted study, we characterized the antigenic landscape of MM by mass spectrometric analysis of frequently, naturally presented HLA ligands of 15 primary MM samples and cell lines (Walz/Stickel et al., Blood, 2015). Comparative profiling of the MM derived HLA ligands and the HLA ligandome of healthy tissues (such as blood, bone marrow, spleen) revealed various strictly MM-associated antigens. This data was here examined for the presence of BCMA-derived peptides. Two HLA class I-restricted peptides were detected in the HLA ligandome of MM, with one showing MM exclusive presentation and which was never identified on any normal tissue according to our HLA

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ligandome database of healthy tissues (135,354 peptides originating from 16,626 source proteins detected in 337 samples from various normal tissues). This HLA-B*18-restricted BCMA ligand $P(BCMA)_{B*18}$ was represented with a frequency of 20% (3/15) in the analyzed myeloma HLA ligandomes.
In order to prove the immunogenicity of our $P(BCMA)_{B*18}$ we performed *in vitro* artificial antigen-presenting cell-based priming experiments with $CD8^+$ cells obtained from healthy volunteers. Tetramer-positive populations with frequencies ranging from 0.1-2.9% of viable $CD8^+$ cells could be observed for all analyzed healthy whole blood donors. Subsequently, functional characterization was performed using intracellular cytokine staining, revealing a TNFα and IFNγ production as well as a CD107a up-regulation in the analyzed tetramer-positive T cells of two healthy volunteers. Moreover, priming experiments using T cells of MM patients and killing assays with peptide-specific T cells are ongoing.
Taken together, we identified and performed an immunological characterization of a naturally presented BCMA-derived peptide, showing exclusive presentation in the HLA ligandome of primary myeloma cells. This BCMA-derived peptide may serve as a feasible target for future T-cell based immunotherapeutic approaches.

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Citrullination: a mechanism to rapidly alert the immune system to cellular stress
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Citrullination is the post-translational modification of arginine to citrulline mediated by Peptidylarginine deiminases (PADs), which are a family of calcium dependent enzymes found in a variety of tissues. Citrullinated proteins are known to play a significant role in the pathogenesis of autoimmune diseases and are presented in complex with MHC class II. Presentation of citrullinated peptides by APCs has been shown to be dependent upon autophagy in addition to PAD activity. Autophagy is also induced by stress in other cells and enables processing of endogenous antigens for presentation on MHC class II molecules. Stressful conditions in the tumor microenvironment induce autophagy in cancer cells to promote their survival. We have previously shown that citrullinated peptides from vimentin are presented on HLA-DR4 in tumor cells via autophagy and mediate strong anti-tumor responses (Brentville et al. 2016). In addition we have demonstrated that these citrulline specific responses can also be mediated through the more common HLA-DP4 allele suggesting a more promiscuous nature for citrullinated peptides. Healthy human donors and cancer patients both show repertoires of CD4 T cells that respond to these citrullinated peptides. In this report we show immunization of HLA-DP4 and HLA-DR4 mice with citrullinated peptides in combination with TLR ligands resulted in generation of strong cytotoxic Th1 responses which protect against tumour challenge. These citrulline specific Th1 responses could be detected rapidly within two days of vaccination suggesting there is a pre-existing repertoire to these citrullinated peptides. In contrast immunization with the citrullinated peptides in a “non inflammatory depot” such as incomplete Freund's adjuvant resulted in T cells secreting IL-10 and no tumour protection. Citrulline specific Th1 responses were able cause regression of large established B16 melanomas within 4 days of vaccination by promoting a pro-inflammatory environment highlighted by increased T cell infiltrate. This suggests that a repertoire of CD4 T cells exists to prevent autoimmunity in the absence of inflammation but that this population can be polarized in the presence of inflammation to rapidly target stressed cells including tumors.

Brentville et al., 2016. Citrullinated vimentin presented on MHC-II in tumor cells is a target for CD4+ T cell-mediated antitumor immunity. Cancer Research 2016 Feb 1;76(3):548-60.

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Citrullinated peptides from self-antigens can be presented by HLA-DP4 and can be targeted for tumor therapy

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Post-translational modifications can be induced in stressed cells and cause them to be recognised by the immune system. One such modification is citrullination where the positive charged arginine is modified to a neutral citrulline. Our previous studies have demonstrated the stimulation of potent CD4 T cell responses to citrullinated vimentin and enolase restricted through HLA-DR4 result in strong anti-tumor immunity. Here we show that two citrullinated vimentin peptides and one citrullinated enolase peptide can also be presented via HLA-DP4, an allele expressed by 70% of the Caucasian population. These citrullinated peptides stimulated CD4 proliferative responses in healthy donors and showed induction of high frequency Th1 responses to citrullinated vimentin and enolase epitopes in HLA-DR4 and DP4 transgenic mouse models. The modification of arginine to citrulline enhanced binding of the some of the peptides to HLA-DR4 and/or HLA-DP4 but made no difference to others, suggesting that citrullination can either enhance MHC binding or TCR recognition. In the presence of TLR4/9 adjuvants the three citrullinated peptides induced strong anti-tumor immunity against established B16 melanoma expressing HLA-DR4 or DP4 (60-100% survival, $p < 0.0001$) and showed significant tumor therapy in the LLC/2 lung cancer models expressing HLA-DR4 or DP4. Since most tumors do not constitutively express MHC class II molecules models were engineered that expressed MHC class II under the control of an IFNγ inducible promoter. Immunisation resulted in 80% survival ($p < 0.0001$) against established B16 tumor expressing inducible DR4 or 90% survival ($p < 0.001$) against established B16 tumor expressing inducible DP4. These studies suggest citrullinated peptides could be used to stimulate strong anti-tumour immune responses in both HLA-DR4 and HLA-DP4 individuals, 25% and 70% of the Caucasian population respectively.

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CD8+ T cell immunity against phosphopeptide neoantigens and its potential use for monitoring checkpoint inhibitor therapy

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Checkpoint inhibition beneficially impacts the survival of patients with many cancers. Checkpoint inhibition leads to a stimulation of the T cell-mediated immune response and this is thought to target mainly mutated peptides. However, many cancers with a low mutational burden have been shown to be highly immunogenic and to respond to CPI therapy and vice versa. Phosphopeptides have emerged as novel candidates for tumor-specific antigens, since dysregulation of signaling pathways in cancer leads to aberrant and augmented protein phosphorylation. In such way modified proteins can

be degraded to generate cancer-specific, MHC class I-bound phosphopeptides (MHC-I-pP). To date we have identified several hundreds of MHC-I-pP by directly sequencing phosphopeptides eluted from MHC molecules with high resolution mass spectrometry mainly for primary liver cancer. But we could demonstrate that MHC-I-pP are not only shared between different tumor samples but also between different cancer entities. Many of the identified MHC-I-pP were derived from proteins, which can be directly linked to important cancer-associated signaling-pathways. MHC-I-pP were displayed mainly on malignant tissue and not on adjacent healthy tissue indicating their tumor specificity. But clinical validation and in vivo immunogenicity investigations are required to fully understand the tumor specificity of these post-translational modified epitopes and their value as potential immunotherapeutic targets.
Therefore we set out to analyze anti-phosphopeptide CD8+ T cell immune responses in healthy donors, patients with chronic liver disease predisposing for cancer and with primary liver cancer. Our data indicated that anti-phosphopeptide immunity develops at a certain age and/or after the development of pre-neoplastic chronic liver disease. This anti-tumor immunity vanished after development of a primary liver cancer. Anti-phosphopeptide immunity was restored again after eradication of the tumor (e.g. post liver transplantation). Interestingly, we were able to detect anti-phosphopeptide immune responses in a fraction of cancer patients receiving CPI therapy although a significant tumor burden was still present. Therefore, immunity against phosphopeptide neoantigens might be re-induced by CPI therapy. In order to answer this important clinical question, we assessed anti-phosphopeptide immunity before and during CPI therapy in a cohort of patients with malignant melanoma as CPI therapy is infrequently used in patients with liver cancer. Our preliminary data reveals a change in the pattern of immunity against phosphopeptide neoantigens after initiation of therapy. The emergence of anti-phosphopeptide immunity will be correlated with clinical response to CPI therapy and other laboratory and immunological biomarkers. In conclusion, our results suggest that MHC-I-pP may be the target of cancer immune surveillance and might represent an attractive target for anti-cancer immunotherapy.

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Unique true predicted neoantigens (TPNAs) correlates with anti-tumor immune control in HCC patients

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A novel prediction algorithm is needed for the identification of effective tumor associated mutated neoantigens. Only those with no homology to self wild type antigens are true predicted neoantigens (TPNAs) and can elicit an antitumor T cell response, not attenuated by central tolerance. To this aim, the mutational landscape was evaluated in HCV-associated hepatocellular carcinoma.
Liver tumor biopsies and adjacent non-tumor liver tissues were obtained from 9 HCV-chronically infected subjects and subjected to RNA-Seq analysis. Mutant peptides were derived from single nucleotide variations and TPNAs were predicted by comparison with corresponding wild-type sequences, non-related self and pathogen-related antigens. Immunological confirmation was obtained in preclinical as well as clinical setting.
The development of such a novel algorithm resulted in a handful of TPNAs despite the large number of predicted neoantigens. Furthermore, TPNAs may share homology to pathogen's antigens and be targeted by a pre-existing T cell immunity. Cross-reactivity between such antigens was confirmed in an experimental pre-clinical setting. Finally, TPNAs homologous to pathogen's antigens were found in the only HCC long-term survival patient, suggesting a correlation between the pre-existing T cell immunity specific for these TPNAs and the favourable clinical outcome.
The new algorithm allowed the identification of the very few TPNAs in cancer cells, and only those targeted by a pre-existing immunity strongly correlated with long-term survival. Only these represent

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the optimal candidates for immunotherapy strategies.

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Selective FcγR engagement by CTLA-4 antibodies results in increased functional activity
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Therapeutic antibodies targeting T-cell co-inhibitory pathways, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1), have emerged as an important class of cancer therapies. Insights into how different IgG isotypes modulate biological activities of antibodies have opened new avenues to potentially enhance their therapeutic effects. For example, Fc-FcγR interactions have been shown to enhance antibody-directed effector cell activities, as well as antibody-dependent forward signaling into target cells via receptor clustering. Here, we describe a novel FcγR-dependent mechanism for antibodies targeting CTLA-4. Our findings suggest that selective Fc-FcγR binding improves the quality of the immune synapse, which in turn modifies apical T cell receptor signaling events to increase effector T cell activity. Our data also suggest that subsets of antigen-presenting cells (APCs), expressing FcγRIV in mice and FcγRIIIA in humans, are important mediators of this effect. Importantly, we find this mechanism to be independent of regulatory T-cell (Treg) depletion. Altogether, we describe a novel mechanism of action that provides a foundation for a new class of Fc-engineered antibodies to enhance antitumor immune responses.

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Mass spectrometry-based antigen discovery expands the cancer immunopeptidome
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Comprehensive knowledge of the HLA peptides presented to T cells is crucial for designing innovative immuno-therapeutics. For example, the identification of mutated HLA peptides has shown great potential in the efficacy of checkpoint blockade therapies; however, many patients with low mutational load do not respond. Therefore, the search for other types of personalized immunogenic antigens beyond neoantigens remains necessary.
To achieve this overall goal, we first developed a mass spectrometry-based immunopeptidomics pipeline that led to substantial improvements in speed, throughput, reproducibility and sensitivity of the HLA extraction process (Chong et al, MCP, 2017). Following this, we characterize a panel of patient-derived primary ovarian, skin and renal cancer cell lines, where autologous T cells are available, by integration of HLA-I and -II immunopeptidomics with proteomics, genomics and transcriptomics studies. Additionally, we employ epigenetic drugs, such as DNA methyltransferase inhibitors (DNMTi) to augment tumor immunogenicity through up-regulation of antigen presentation from normally

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silenced genes, e.g. cancer-testis antigens and other potential antigens of non-conventional origin. We identified with this mass spectrometry approach patient-specific tumor-associated, DNMTi-induced and non-conventional HLA-I and -II peptides, as well as neoantigens. Currently, we validate their detection both at the computational and at the MS/MS level. The assessment of immune-recognition with in-vitro T cell-based assays is ongoing.
In conclusion, to better understand the nature of tumor immunogenicity and the different antigen types that would generate clinically relevant targets, we are performing an exploratory approach to gain deep insight into the immunopeptidome and the antigenic landscape over various cancer types.

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Survivin, WT1 and SSX2: new targets for the immunotherapy of adult B-acute lymphoblastic leukaemia

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Acute lymphoblastic leukaemia (ALL) is a form of leukaemia characterized by excess lymphoblasts in the bone marrow.The most effective treatment to date is allogeneic stem cell transplant which can improve overall survival rates and may in part be due to a 'graft-versus-leukaemia' effect. However few of the cancer antigens have been identified in adult B-ALL which could act as targets for immunotherapy. We have examined a total of 12 different antigens (BCP-20, G250, HAGE, END, NY-ESO-1, PASD1, p68 RNA helicase, SSX2, SSX2IP, survivin, tyrosinase and WT1) in adult B-ALL patient samples and healthy volunteers. We found that only survivin and WT1 were expressed in B-ALL patient samples (7/11 and 6/11, respectively) but not normal donor control samples (0/8).Real-time quantitative (RQ)-PCR showed that survivin was the only antigen whose transcript exhibited significantly higher expression in the B-ALL samples (n= 10) compared with healthy controls (n= 4)(p= 0.015). Surprisingly SSX2 protein expression was demonstrated by ICC in all patient samples examined, suggesting a disconnect between detectable RNA transcription and protein translation of this cancer-testis antigen. To determine whether these findings were supported by the analyses of a larger cohort of patient samples, we performed metadata analysis on an already published microarray dataset (GSE38403). We found that only Survivin (p=0.013, ANOVA) and Endoglin (p=0.015, ANOVA) were frequently over-expressed in B-ALL patient samples (n=215) versus normal PreB cells (n=12). Of the twelve candidate genes investigated only p68 DNA helicase, SSX2IP, Survivin and WT1 showed significant differences in expression when compared across individual cytogenetic groups. Elevated END or survivin expression was significantly associated with the t(9;21) translocation while p68 DNA helicase, SSX2IP, Survivin and WT1 expression were associated with different 11q23 /MLL abnormalities. We did examine whether there was a correlation between OS and event free survival (EFS) with the expression of each gene but none achieved significance and the closest to achieving significance was SSX2 interacting protein (SSX2IP) with an association with OS (p = 0.078). Concurrent antibody specific profiling was performed on sera samples from nine adult B-ALL patients and nine age and sex-matched healthy donor controls. Signals from 9,000 peptides were analysed on the ProScanArray using ProtoArray® Prospector v5.2 software. The mean value and standard deviation of each signal was calculated to produce a z-score and the six most promising antigens identified. All have been shown to be associated with solid tumour development and their exploration in adult B-ALL is now being pursued.

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Cell-intrinsic and spatially distant tumor PD-L1 alters local and global anti-tumor immunity and responses to anti-PD-L1 or anti-PD-1 through novel chemokine and TIL effects

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Programmed death ligand 1 (PD-L1) is expressed on many tumors and inhibits anti-tumor T cells through T cell programmed death (PD)-1 engagement. Tumor PD-L1 predicts anti-PD-L1 treatment outcomes through incompletely understood mechanisms and is an imperfect treatment response marker. We used naturally PD-L1^{+} murine B16 melanoma (ctrl) and made PD-L1^{KO} B16 using CRISPR/Cas9. In subcutaneous challenges of wild type BL6 mice, PD-L1^{KO} B16 grew faster when ctrl B16 was on the opposite flank (*trans*). By contrast, ctrl B16 grew slower when *trans* to PD-L1^{KO} B16. Anti-PD-L1 and anti-PD-1 slowed ctrl but not PD-L1^{KO} B16 tumors as expected. However, when PD-L1^{KO} was *trans* to ctrl, anti-PD-L1 and anti-PD-1 now also slowed PD-L1^{KO} growth. These effects appeared independent of host PD-L1, ADCC or microbial influences. Anti-PD-L1 increased CD3^{+} T cell infiltration into ctrl and PD-L1^{KO} tumors similarly in vivo, but increased natural killer (NK) cell numbers and functions (*e.g.,* CD107a, IFN-g) in PD-L1^{KO} tumors greater than in ctrl tumors. CD4^{+} and CD8^{+} T cells chemotaxed to PD-L1^{KO} slightly greater than to ctrl B16 cells, but NK cells migrated 2-fold more to PD-L1^{KO} vs ctrl B16 cells. PD-L1^{KO} tumors produced more CCL2 than ctrl in vitro and in vivo, and this CCL2 induced CCR2^{+} NK chemotaxis *in vitro*. Tumor PD-L1 controlled T cell migration through tumor CXCL10/T cell CXCR3. Both NK cells and T cells were required for optimal *trans* responses to anti-PD-L1 in vivo. In wild type mice, anti-CCL2 reduced growth of *trans* PD-L1^{KO} tumors > ctrl, and reduced anti-PD-L1 efficacy against PD-L1^{KO} > ctrl tumors. In CCR2^{KO} mice, anti-PD-L1 efficacy was retained against trans ctrl B16, but abolished against PD-L1^{KO} *trans* tumors. Similar trafficking or chemokine effects were seen in MB49 bladder cancer and 4T1 breast cancer cells. Thus, tumor PD-L1 alters immune infiltrates and anti-PD-L1 efficacy through novel chemokine effects. Our models help understand anti-PD-L1 (and likely anti-PD-1) responses based on tumor PD-L1 expression and can define ways to improve immunotherapy for PD-L1 null tumors.

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A novel aryl hydrocarbon receptor antagonist (PX-A24590) with anti-tumor activity in a syngeneic mouse pancreatic cancer model

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The aryl hydrocarbon receptor (AhR) is a ligand-controlled transcription factor that is widely known for mediating the toxicity and tumor-promoting activities of halogenated hydrocarbons (like Dioxin, TCDD) and polycyclic aromatic hydrocarbons (e.g. Benzo(a)pyrene and 3-Methylcholanthrene). Other ligands include metabolites produced by commensal microorganisms on the skin and in the gut and are known to modulate the transcriptional activity of AhR in different immune cells (e.g. ILC type 3 in the gut) and epithelial cells, thereby balancing the immune system´s response towards these microorganisms. In recent years, endogenous L-Tryptophan metabolites, such as L-Kynurenine and Kynurenic acid, that are produced under control of the Indole-2,3-Dioxigenases IDO1 and TDO2 pathways, were shown to activate the AhR.
Constitutive activation of the IDO1/TDO2/AHR pathway and nuclear AhR protein accumulation is frequently observed in different tumor types, which is thought to be linked to the observed diminished anti-tumor immune response. It is believed that secreted AhR activating ligands aid in reducing the pressure exerted on tumors by the immune system through increasing the numbers and function of regulatory T cells and reducing the numbers and function of cytotoxic CD8^{+} T cells. Interestingly,

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inhibition of IDO1 by Epacadostat has recently shown remarkable activity to support the anti-tumor efficacy of the anti-PD-1 checkpoint inhibitor Keytruda in metastasizing melanoma.
In order to relieve AhR-mediated immune-suppression, Phenex Pharmaceuticals initiated a program to identify small molecule AhR antagonists to block downstream signaling of the AhR due to activating endogenous ligands. Towards this, we have identified a novel AhR antagonist, PX-A24590, which showed strong antagonistic activity against human AhR in a cell based CYP1A1 promoter-driven luciferase reporter assay in HepG2 cells. PX-A24590 displays good oral bioavailability and low clearance in mice. In C57BL/6 mice transplanted with syngeneic Panc02 pancreatic tumor cells, we were able to demonstrate anti-tumor efficacy with three different oral doses of PX-A24590 compared to an effective dose of the IDO1 inhibitor Epacadostat.

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Comprehensive characterization of IL-15-based tumor targeted Immunocytokines
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Cytokines are promising drugs for anti-cancer treatment as they modulate immune responses. Yet, administration of cytokines is often hindered by dose-limiting toxicities preventing their use as effective modulators. Particularly interleukin-15 (IL-15), as a potent stimulator of NK and CD8 T cells, is an attractive cytokine for cancer therapy. IL-15 induces high levels of anti-tumor cytotoxicity when used in combination with common tumor targeting antibodies *in vitro* and *in vivo*. However, by specifically targeting the tumor using an IL-15 based immunocytokine immune cells are preferentially activated at the local tumor site thereby increasing the potency of common tumor targeting antibodies.
As a prerequisite for safe and effective immunocytokine therapy highly specific tumor targets are necessary. One promising tumor antigen is TA-MUC1, a novel carbohydrate / protein mixed epitope on the tumor marker MUC1 that is virtually absent from normal cells. TA-MUC1 shows a broad distribution among epithelial cancers of different origin and is also present on metastases and cancer stem cells underpinning its broad therapeutic potential. Based on our anti-TA-MUC1 IgG1 PankoMab-GEX we developed an immunocytokine platform consisting of different constructs to analyze the influence of IL15 potency, tumor targeting via TA-MUC1 and additional Fc-mediated immune cell binding. Three types of IL-15 with varying potency were tested. Furthermore, we generated Fc-silenced immunocytokines to analyze the impact of a functional Fc part especially on NK cell activation and cytotoxicity. In addition, we developed untargeted immunocytokines to point out the influence of the TA-MUC1 antigen binding.
Functional characterization of these immunocytokines involved *in vitro* analysis of immune cell activation, proliferation, cytotoxicity and cytokine release. By using this broad screening approach, we could show that our immunocytokines induce higher cytotoxicity against tumor cells and increase activation and proliferation especially on NK, NKT and CD8 T cells compared to the common tumor targeting IgG1 originator antibody. The mediated cytotoxicity was strongly dependent on TA-MUC1 antigen binding, IL-15 potency and the functional Fc terminus.
The study emphasizes the advantage of comprehensive construct screening for immunocytokine lead candidate identification and in particular highlights TA-MUC1 as a promising antigen for development of immunocytokines as the untargeted immunocytokines did not achieve comparable efficacy.

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Engineering novel antibody-based therapeutics for targeting of tumor endothelial marker 1 (TEM1)
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Recent advances in immunotherapy have revealed the potential of human T cells to participate in the control of tumor growth. One clinically validated paradigm for harnessing the cytotoxic potential of T cells involves the use of bispecific antibody-derived molecules comprising a tumor-targeting moiety together with an associated 'bridging' anti-CD3 recognition domain. Alternatively, T cells can be equipped with a chimeric antigen receptor (CAR) by combining a tumor antigen specific single chain variable fragment (scFv) with co-stimulatory signaling domains. Both strategies have been applied successfully to treat hematological malignancies, but immunotherapeutic targeting of solid tumors remains challenging, partly due to insufficient tissue penetration. This obstacle could be bypassed by selectively targeting the supporting tumor neo-vasculature. Tumor endothelial marker 1 (TEM1, Endosialin) is specifically upregulated in the stroma and vasculature of many solid tumors, while being largely undetectable in healthy tissues.
In the present study, we aim to explore the therapeutic potential of redirecting T cells to selectively kill TEM1 expressing tumor neo-vasculature, using *de novo* discovered scFv antibodies.
To this end, we isolated a panel of fully human scFv molecules against TEM1 from a large, naïve phage display library. Extensive screening, biophysical characterization, affinity measurements and functional binding assays identified a set of specific scFv binders to both human and murine TEM1. Clones with desirable properties were subsequently fused to an anti-CD3 recognition domain in order to engineer T cell engaging bispecific mediators. In this context, we have also reformatted candidate scFvs in a trivalent engager format that permits bivalent or biparatopic tumor targeting for increased design flexibility. Alternatively, TEM1-specific scFv candidates were fused to CD28-CD3zeta co-stimulatory signaling domains and introduced into human T cells to generate CAR T cells that may selectively target tumor neo-vasculature. Currently, we are evaluating the efficacy of different bispecific antibody formats in redirecting T cells to TEM1 expressing target cells, as well as the cytotoxic potential of T cells transduced with different anti-TEM1 CARs. Moreover, our novel antibodies are being evaluated for molecular tumor imaging and as antibody-drug conjugates.
Together, we have developed a diverse set of fully human scFv antibodies directed against TEM1, which are currently being harnessed in the context of T cell engaging bispecifics and CARs in order to explore the potential of TEM1 as a target for cancer immunotherapy.

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RIG-I agonist acts synergistically with PD1-checkpoint blockade as combinatorial immunotherapy in a model of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is a malignancy of the liver characterized by poor prognosis and unsatisfactory 5-year survival rate. Triphosphate-RNA (3p-RNA) is recognized by the innate immune receptor RIG-I and triggers a type-I IFN-mediated immune response and an immunogenic form of cell death in tumor cells. Here, we identified RIG-I as a novel target in HCC and evaluated its therapeutic efficacy as single therapeutic agent and in combination with immune checkpoint inhibition in a model of HCC.
RIG-I expression levels and validation as targetable receptor in human HCC were assessed by immunohistochemistry analysis of tissue microarrays of human HCC biopsies. Furthermore, RIG-I pathway activation following 3p-RNA treatment was evaluated *in vitro* in human and murine HCC cell

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lines. Lastly, the immunotherapeutic efficacy of RIG-I activation as a single agent and as in combination with immune checkpoint inhibition was tested in an orthotopic HCC mouse model. RIG-I activation and functional activity following transfection of 3p-RNA was confirmed by phosphorylation of IRF-3, production of IFN-b, CXCL10, and the induction of tumor cell death in both human and murine HCC cell lines. In an orthotopic HCC mouse model, treatment with 3p-RNA significantly prolonged survival, and the therapeutic efficacy proved to be dependent on $CD8^+$- and $CD4^+$ T cells. Depletion of the essential downstream adapter protein MAVS or the IFN-receptor IFNAR1 in the host did not influence therapeutic outcome implicating a tumor intrinsic effector function of 3p-RNA. While anti-PD1 checkpoint inhibition administered as a single agent did not show survival benefits, anti-PD1 used in a combinatorial regiment with the RIG-I agonist 3p-RNA dramatically boosted therapeutic efficacy prolonging and increasing survival.
In conclusion, RIG-I represents a relevant and promising target for HCC immunotherapy. Furthermore, the survival benefits of RIG-I activation are dramatically enhanced when combined with checkpoint inhibition, delineating prominent therapeutic strategies in HCC and possibly in other tumor models.

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The immunopeptidomic landscape of primary *versus* recurrent disease in glioblastoma

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Glioblastoma is the most frequent and most aggressive primary tumor of the central nervous system. Scientific knowledge has not yet sufficiently translated into clinical benefit, although extensive research has been conducted. Owing to deep infiltration into surrounding benign tissue, complete surgical resection is impossible and glioblastoma inevitably recurs. Especially at recurrence, the lack of evidence-based therapeutic options results in poor clinical outcome. In this setting, immunotherapeutic approaches, especially T cell-based strategies, seem very promising. Importantly, such immunotherapeutic intervention typically takes place after standard radiochemotherapy contributing to clonal evolution and potential hypermutation in glioblastoma and may thereby drastically alter the antigenic landscape of the tumor.
We employ a multi-omics approach including HLA peptidomics, exome and RNA sequencing to define candidate targets for glioblastoma immunotherapy. By immunoaffinity chromatography and tandem mass spectrometry, we profiled the immunopeptidome of seven patients with 22,273 unique HLA class I and 20,579 unique HLA class II peptide identifications in total. This revealed six antigens with frequent and exclusive HLA presentation at recurrence (43% to 57% positive samples) and nine frequently and exclusively presented on primary tumors (57% to 86% positive samples). Label-free quantitation of HLA class I ligands was possible for six patients, delineating significantly modulated peptides in recurrent *versus* primary disease. Upon recurrence, 13% of the patients' immunopeptidomes showed significant up- or down-modulation, with corresponding source proteins being shared between up to four patients. Established glioblastoma-associated antigens were identified, but were not tumor-exclusive, when considering an in-house benign database. Thus, we defined a (novel) set of ten tumor-associated antigens robustly presented on both primary and recurrent tumors (50% to 64% positive glioblastoma samples in total).
So far, exome and RNA sequencing have been performed for three patients, identifying one with a

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hypermutation phenotype at recurrence (324 *versus* 12,012 somatic variants). Of note, a substantial increase in the proportion of missense mutations - potentially giving rise to neoantigenic peptides - was observed.
The present work provides insight into the immunopeptidomic landscape of glioblastoma and its modulation in the context of clonal evolution during progression from primary to recurrent disease. Using this strategy, we aim to define (novel) potential targets with robust antigen presentation patterns for the immunotherapy of recurrent glioblastoma.

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Tumor antigen-dependent T cell activation and tumor localization induced by a novel 4-1BB x 5T4 ADAPTIR™ bispecific antibody

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4-1BB (CD137; TNFRSF9) is an activation-induced costimulatory receptor and a key regulator of immune responses expressed primarily on antigen-specific activated CD8 T cells. Upon 4-1BB stimulation, CD8 T cells undergo enhanced proliferation and increased survival, intensify their cytolytic activity and enhance their IFN-γ production. The ability to induce potent anti-tumor activity by stimulating 4-1BB on tumor specific T cells makes 4-1BB-targeting immunotherapeutic strategies very attractive. However, clinical development of a strongly agonistic monospecific 4-1BB antibody has been hampered by dose-limiting hepatic toxicities.
Here we present a novel 4-1BB x 5T4 targeting bispecific antibody, ALG.APV-527, designed to induce potent tumor specific CD8 T cell activation, while minimizing unwanted systemic toxicities. ALG.APV-527 contains binding domains specific to both the co-stimulatory receptor 4-1BB and to 5T4, a tumor associated antigen expressed on multiple solid tumors. ALG.APV-527 only activates 4-1BB when 5T4 is engaged on tumor cells. This feature localizes the immune stimulatory effects of ALG.APV-527 to the tumor microenvironment where both 4-1BB and 5T4 are highly expressed. Therefore, ALG.APV-527 has the potential to be a potent anti-cancer therapeutic agent with an improved safety profile. Furthermore, as 5T4 is widely expressed on a variety of tumor types, ALG.APV-527 is an attractive drug candidate for many different solid tumor indications.
The dual binding domains of ALG.APV-527 originate from the Alligator-Gold® human scFv library (Alligator Bioscience AB), and were further optimized and developed for use in the bispecific ADAPTIR format (Aptevo Therapeutics Inc.). The merger of these elements generated a highly functional, tumor-targeting bispecific antibody featuring enhanced potency, increased binding affinity, optimal stability, and good manufacturing properties.
In vitro, ALG.APV-527 increases CD8 T cell effector function, as measured by IFN-γ production only in the presence of the tumor antigen 5T4, while in the absence of tumor antigen it is essentially inert. Additionally, ALG.APV-527 demonstrates high potency in a 4-1BB NF-κB reporter assay in the presence of 5T4. Furthermore, by using a B16 melanoma twin tumor model where mice were inoculated with one 5T4-positive tumor and one 5T4-negative tumor, we could demonstrate that the bispecific antibody preferably localized to 5T4-positive tumors in an antigen-dependent manner.
In conclusion, we have developed a novel 4-1BB x 5T4 ADAPTIR bispecific antibody that, based on preclinical data, has the potential to be a unique anti-cancer therapeutic agent for the treatment of a variety of 5T4-expressing solid tumors with a high unmet medical need.

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Decoding the HLA immunopeptidome: Elucidating patterns of HLA allotype association and malignant cells

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The human leukocyte antigen (HLA) immunopeptidome constitutes a showcase of intra- and intercellular processes capable of modulating the adaptive immune response. Despite highly polymorphic HLA molecules and various diverse cell types distributed over different tissues, the immune system can selectively distinguish between healthy and diseased cells. In this study, we employ machine learning to dissect the immunopeptidome for patterns of HLA allotype association as well as tissue disease status.
To that end, we base our analysis on an in-house database containing 183 malignant (20 different tumor entities) and 215 healthy HLA typed samples, generated using HLA immunoprecipitation and subsequent liquid chromatography tandem mass spectrometry analysis of eluted peptides. The complete dataset was randomly divided into a training and test set. The test set included each HLA allotype and a similar distribution of sample dignities as the training set. Using machine learning, HLA allotype and malignancy characteristic HLA ligands in the HLA immunopeptidome were decoded.
In the test set, our approach enabled a correct prediction of 89% of the HLA allotypes including samples ranging in the number of peptide identifications between a few hundred up to thousands of HLA ligands. This result was corroborated using external, published primary tissue HLA ligandome datasets achieving a 94% correct prediction rate. In a similar way the dignity of the tissue samples could be predicted correctly for 85% of the malignant samples in the test set and 86% in the external dataset. A closer look into the sets of HLA ligands defining patterns for HLA allotypes revealed allotypic peptides fitting to established peptide binding motifs. In case of cellular malignancy defining peptides are frequent peptides with either high malignant or healthy tissue occurrence as well as published tumor specific HLA ligands.
Here we describe a machine learning approach to mine immunopeptidomic data for patterns of HLA ligands specific for different HLA allotypes as well as tissue disease state. This approach can be utilized for many purposes for example to improve HLA immunopeptidomics as well as T-cell assays or to guide the development of targeted immunotherapies. Moreover, these results illustrate the predictive potential of HLA immunopeptidomic data and envisage the use in the development of biomarkers for different cellular disorders.

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Targeting the signal peptide in MMTV-associated breast cancer

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In 2015 the World Health Organization reported 571,000 deaths from breast cancer worldwide. According to the American Cancer Society, 252,710 new cases of invasive breast cancer and 40,610 breast cancer deaths were expected to occur among USA women in 2017.
Mouse Mammary Tumor Virus (MMTV) is a Beta-retrovirus that causes mammary carcinoma and lymphoma in mice. An increasing body of evidence in recent years supports the involvement of MMTV in over 30% of human sporadic breast cancers.
We have identified the signal peptide of the envelope precursor protein of MMTV (named by us MMTV-p14, or p14 for short) as a novel target for immune therapy of MMTV-associated breast cancer,

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based on the following characteristics:
1) It is expressed on the cell surface , as well as in nucleoli of murine lymphomas, mammary carcinomas and human breast cancer cells that contain (or ectopically express) MMTV sequences.
2) p14 is immunogenic, insofar as injection of the purified molecule into mice (using different adjuvants) induces specific antibody and T-cell mediated responses. We have exploited this trait for preventive vaccination against murine tumors that harbor MMTV.
3) Monoclonal anti-p14 antibodies as well as T-cells, isolated from mice primed and challenged with p14, were successfully used in *in vivo* immune therapy of MMTV-bearing murine tumors.
4) Interestingly, p14 has been found in sera of syngeneic mice challenged with murine mammary carcinoma or lymphoma cells that contain MMTV, suggesting shedding of this 98 amino-acid protein (or peptides thereof) as a putative immune evasion mechanism.
5) Being a phosphoprotein, p14 functions, either in an oncogenic or anti-oncogenic capacity *in vivo*, depending on its phosphorylation status.
Taken together, the above findings constitute a novel strategy towards preventive vaccination, detection and treatment of MMTV-associated breast cancers based on p14 (signal peptide) targeting.

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Development of an optimized scaffold for bispecific T cell receptor therapeutics
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Bispecific T cell receptor (TCR)-antibody fusion proteins against tumor-specific targets represent a promising class of cancer therapeutics. The utilization of a TCR moiety is a major advantage of these molecules as it allows targeting of human leucocyte antigen (HLA)-bound peptides derived from virtually all proteins of the tumor cell regardless of their extracellular or intracellular location. Immatics is developing TCR bispecifics against tumor associated peptide-HLA targets, which have been identified and validated by its proprietary target discovery engine XPRESIDENT®. Immatics has further established a portfolio of technologies to discover and engineer TCRs originating from the natural repertoire of human donors. After affinity maturation of single chain TCRs (scTv), the mutant scTv candidates displaying enhanced stability and affinity serve as building blocks for the generation of soluble and highly potent bispecific T cell receptor therapeutics.
Here we present the development efforts to generate an optimized scaffold for the construction of such bispecific TCR molecules. Seven different TCR-antibody fusion scaffolds with various variants thereof were designed utilizing an affinity maturated scTv specific for the HIV-derived SL9 peptide (SLYNTVATL) presented on HLA-A*02. For engagement of T cells, two different antibody-derived domains were tested. All candidate molecules were purified from CHO-supernatants using conventional two-column chromatography and subjected to thoroughly *in vitro* testing including stress studies. The scaffolds were ranked according to developability characteristics including productivity and stability as well as efficacy and safety data to finally determine the molecular architecture of Immatics' bispecific T cell receptor therapeutics platform.

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Rapamycin and Zoledronic Acid strongly inhibit growth of advanced murine hepatocellular carcinoma via activation of innate and adaptive immunity
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Treatment options for hepatocellular carcinoma (HCC) remain limited. Activation of the cancer immune microenvironment opens novel therapeutic opportunities to control tumor progression and block metastatic spread. We assessed single and combined treatment of experimental HCC with Zoledronic Acid (ZA) and Rapamycin (RA), two drugs that display myeloid cell regulating potential.
Methodology: Mdr2(Abcb4)-/- mice were injected intraperitoneally with diethyl-nitrosamine DEN (10µg/g of bw) at the age of 5 days, followed by 0.05% phenobarbital in drinking water starting at the age of 3 weeks. This syngenic mouse model replicates key features of human HCC, with a genetic defect found in man and an external carcinogen. Mice were treated with vehicle,
(ZA thrice a week as IP injection 100µg/kg), RA (trice a week as oral gavage 5mg/body weight), or a combination of ZA and RA from age 5-6 months. After 6 months tumor volume and number of nodules were counted. 50% of livers were digested and CD45+ immune cells subjected to multicolour fluorescence assisted cell sorting using antibodies to CD11b, CD11c, Ly6C, Ly6G, CD86, CD4, CD8, CD25, CD3, CD90.2, CD206, F4/80, MHC-II, NK1.1, Foxp3 and CD19. qPCR and IHC were performed for target molecules of interest.
Results: Treatment with RA>ZA significantly reduced tumor growth. The combination of ZA and RA synergistically reduced the volume and number of HCC foci by 90 and 85 % respectively ($p< 0.0001$). The combination significantly reduced the population of M0- ($CD45^+CD11b^+LY6G^-F4/80^+$) and M2 macrophages ($CD45^+CD11b^+LY6G^-F4/80^+CD206^+$), and of myeloid derived suppresser cells ($CD45^+CD11c^+LY6C^{high}$), representing central tumor promoting myeloid cell populations, while Myeloid derived dendric cells ($CD45^+CD11c^{high}$ $CD11b^+$ $CD86^+$) that promote anti-cancer immunity were significantly upregulated. In parallel, total CD4+ T cells and especially CD4+CD25 regulatory T cells were significantly suppressed, while CD8+T cells were significantly increased in the combination treatment vs the untreated group . Changes in immune cell populations were confirmed by transcript patterns and IHC. Ki-67-positive cancer cells were nearly undetectable, and IHC for CD68 and YM-1 confirmed a dramatic shift from tumour associated M2 to M1 macrophages. The combination of ZA and RAPA significantly downregulated TAMs and MDSC markers like CSF-1, VEGF, TGFβ1, Cox2, HIF1α, CCL2, CCL3, CCL5 and CCL17 that attract circulating monocytes into the tumour stroma.
Conclusion:
1. Combination therapy of RAPA and ZA polarize myeloid (and T cells) towards a robust anti-HCC response;
2. Their combination is synergistic in an optimized syngenic model of murine HCC;
3. Both drugs have proven safety for other indications, and clinical studies in patients with HCC are warranted.

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Cytomegalovirus stimulates angiogenesis and emerges as a druggable target in glioblastoma
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Objective: Cytomegalovirus (CMV) has been linked to glioblastoma for over a decade. However, mechanisms how CMV affects tumour growth *in vivo,* are poorly understood. Here we identify a novel regulatory mechanism, involving the cross-talk between infected tumour cells and vascular pericytes to promote angiogenesis in glioblastoma. We demonstrate that treatment with the antiviral drug Cidofovir prolongs survival in a syngeneic mouse glioblastoma model.
Methods: *In vitro* CMV expression in mouse and human glioblastoma samples were analysed. RNAseq and functional assays were used to investigate tube formation and vascular development. *In vivo* treatment experiments are performed using a new CMV latent, orthotopic mouse glioblastoma model.
Results: Immunostaining of patient samples identified viral antigens expressed in multiple cellular compartments including the novel observation of co-localization with pericytes. RNAseq of human brain vascular pericytes (HBVPs) and glioblastoma stem-like cells (GSCs) revealed upregulation of

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proangiogenic cytokines after CMV infection. Conditioned medium derived from CMV infected GSCs/HBVPs led to the establishment of larger (160%, p< 0.0001) and more complex (number of junctions 9.5 (control) vs. 21 (CMV), p< 0.0001) tube formation in human brain microvascular endothelial cells (HBMEC). Platelet derived growth factor DD (PDGF-DD), secreted by GSCs after CMV infection, was identified as a driver of HBVP migration and angiogenesis. Orthotopic injection of Gl261fluc murine glioblastoma cells in C57BL/6 mice harbouring a latent CMV infection caused intratumoural virus reactivation and shortened survival rates (30d vs 43d, p=0.0004). Increased area (p=0.0004) and length (p=0.001) of tumour vessels was found in CMV latent mice. Furthermore, CMV infected tumours showed significantly higher numbers of infiltrating- and blood vessel-associated pericytes. Treatment with the antiviral drug Cidofovir reduced the tumour vasculature and increased the survival of CMV latent mice (mean: 36d, p = 0,0001).
Conclusion: In patient-derived tumour samples CMV co-localizes with the perivascular niche. Tumour growth is accelerated in CMV latent mice, driven by pro-angiogenic paracrine signalling. Secretion of PDGF-DD is associated with increased pericyte recruitment and consecutive angiogenesis. Antiviral treatment prolonged survival after tumour injection in CMV latent mice and might represents a novel therapeutic option in glioblastoma.

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Immunopeptidomic profiling of triple negative breast cancer identifies potential immunotherapy target antigens
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The collection of major histocompatibility complex (MHC) presented peptides, or immunopeptidome, provides in depth information on cell status and function. This is particular interesting in the case of cancers in which a proportion of peptides are produced by dysregulated or mutated genes, hence are specific to cancerous cells and are potential targets for various immunotherapeutic development. We analysed the immunopeptidome landscape of 6 HLA-A2-positive breast cancer patients by liquid chromatography tandem mass spectrometry (LC-MS/MS) and identified a total of 19,675 peptide sequences from cancer and adjacent normal tissues. A number of tumour-specific sequences predicted to bind HLA-A*0201 were identified in 5 patients for which adjacent normal tissue controls were analysed in parallel. We further shortlisted the most relevant tumour-specific source proteins that are presented across the cohort and identified antigens with the highest tumour-specific presentation coverage. Such antigens could be prioritized for future development of T cell mediated breast cancer therapy.

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The CTLA-4 x OX40 bispecific antibody ATOR-1015 induces anti-tumor effects through tumor-directed immune activation
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ATOR-1015 is a CTLA-4 x OX40 bispecific immune activating antibody developed for tumor-directed immunotherapy. ATOR-1015 binds both targets simultaneously, promoting cell-cell interactions expected to enhance the immuno-stimulating effect of the compound. The mode of action of ATOR-1015 is thought to be a combination of regulatory T cell (Treg) depletion and effector T cell activation. It can be seen as a next generation CTLA-4 antibody with tumor-directed activity and augmented Treg

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depletion.
The ability of ATOR-1015 to induce ADCC of human Tregs is demonstrated in a reporter assay using FcγRIIIa-expressing cells and in an LDH release assay with allogeneic NK cells. Further, ATOR-1015 induces activation of T cells in the presence of CTLA-4 or FcγR crosslinking. A superior effect of ATOR-1015 in terms of Treg depletion and T cell activation is demonstrated compared to the combination of monotargeting antibodies.
Syngeneic tumor models *in vivo* using human transgenic mice cross-reacting with both targets demonstrate that ATOR-1015 reduces tumor growth and prolongs survival. Further, treatment synergistically increases the intratumoral $CD8^+$ T cell/Treg ratio compared to the monospecific counterparts, without affecting systemic T cells. ATOR-1015 induces both depletion of intratumoral Tregs and an increase and activation of $CD8^+$ T cells.
In conclusion, ATOR-1015 is a next generation CTLA-4 antibody with tumor-directed activity with augmented Treg depletion. It is currently in GLP manufacturing of clinical material and will start clinical trials during the second half of 2018.

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Anticancer immunity mediated by the novel hexavalent human GITR agonist HERA-GITRL is conveyed by activating T cells without affecting Treg cells

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Key immune regulators belong to the tumor necrosis factor receptor superfamily (TNFR-SF). Glucocorticoid-induced TNFR-related protein (GITR, TNFRSF18, CD357), a TNFR-SF member, is a co-stimulatory receptor that increases anti-tumor T cell activation. Based on Apogenix hexavalent TNFR-SF agonists (HERA) technology platform, we created the fully human hexavalent GITR ligand fusion protein HERA-GITRL intended for T cell costimulatory approaches in immuno-oncology (IO) therapy. HERA-GITRL is composed of a trivalent GITRL-receptor-binding-domain fused to an IgG1-derived silenced Fc-domain serving as dimerization scaffold. Because of the unique design of the silenced Fc-domain, HERA-GITRL allows the study of pure GITR agonism in contrast to Fc-mediated mixed modes of action. Here we report *in vitro* and *in vivo* properties of our novel HERA-GITRL construct.
For functional characterization of HERA-GITRL *in vitro*, human immune cells isolated from healthy-donor blood were profiled by multicolor flow cytometry and real-time cell analysis (RTCA). Stimulation of unfractionated human T cells or purified naïve CD4+ T cells by anti-CD3 antibody was further augmented by HERA-GITRL. This effect was accompanied by increased proliferation, differentiation and elevated levels of TNF-α and IFN-γ. In line with these findings, the murine surrogate mmHERA-GITRL enhanced antigen-specific clonal expansion of both CD4+ (OT-II) and CD8+ T (OT-I) cells *in vivo*. In contrast to anti-GITR antibodies, HERA-GITRL showed no functional effects on regulatory T cells (Treg) either in mono-culture or in co-culture with various ratios of responder T cells (Tresp cells). Specifically, there were no changes in proliferation, survival or cytokine production by Treg cells treated with HERA-GITRL. However, HERA-GITRL prevented Treg-mediated suppression and boosted the proliferation of Tresp cells co-cultured with Treg cells at various ratios. Importantly, HERA-GITRL mediates T cell activation and thereby increases *in vitro* tumor cell killing by PBMCs. Finally, mmHERA-GITRL showed *in vivo* anti-tumor efficacy as a single agent in a subcutaneous syngeneic colon cancer model (CT26wt) in mice.
By clustering six receptor chains in a spatially well-defined manner, HERA-GITRL induces potent agonistic activity without being dependent on additional Fc-mediated crosslinking. The anti-tumor effect of most anti-GITR antibodies is dependent on a fully functional Fc domain and is generally mediated by depletion of Treg cells. HERA-GITRL boosts antigen-specific T cell activity and shows anti-tumor efficacy while having no effect on Treg cells. This property of HERA-GITRL makes it particularly suitable for IO-combination therapies. The HERA concept has also been successfully

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translated to HERA-TRAIL (now in Phase I), -CD40L, -CD27L, -OX40L, -4-1BBL and -LIGHT.

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KY1055; a novel ICOS/PD-L1 bispecific antibody, enhances T cell activation and delivers potent monotherapy anti-tumour responses in vivo

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Antibodies blocking the PD-1/PD-L1 axis have achieved significant breakthroughs in the treatment of hitherto untreatable cancers. However, despite the highly encouraging progress that has been made, multiple tumour types remain poorly responsive to monotherapy. The emerging picture is that combination with other therapies is required to improve response rates and/or the durability of responses. We hypothesize that additional benefit could be achieved by combining anti-PD-L1 with another modulator of T cell activation. Inducible T-cell costimulator (ICOS) is expressed on activated T cells such as T_{Eff} and T_{Reg} cells present in the tumour microenvironment (TME). ICOS agonism has been demonstrated to improve T_{Eff} cell survival and IFNg release, resulting in activation of T cell-dependent anti-tumour activity. In addition, the high expression of ICOS observed on T_{Reg} cells in the TME allows these highly immunosuppressive cells to be selectively depleted when using the appropriate antibody format.
With the aim of targeting both ICOS and PD-L1, we have developed a bispecific mAb (KY1055) as a human IgG_1 that consists of fully human Fab arms targeting ICOS and a modified Fc (Fcab) that retains FcgR and FcRn engagement and additionally contains binding sites to PD-L1. The resultant mAb^2 demonstrates potent (single digit nM) binding to ICOS via the Fab arms and similar low nM affinity binding to PD-L1 via the Fcab. Furthermore, in vitro studies demonstrate that KY1055 can bridge together ICOS- and PD-L1-expressing cells and can activate ICOS signalling via this cross-presentation. Functionality of the Fcab was validated by demonstrating PD1-PD-L1 blockade resulting in the up-regulation of IFNγ in monocyte : T cell co-culture assays. We also have confirmed the ability of KY1055 to deplete $ICOS^{high}$ cells in vitro by ADCC.
In vivo, we confirmed the anti-tumour efficacy of KY1055 in several syngeneic models that are poorly responsive to the respective monotherapies and further demonstrate improved anti-tumour efficacy when combined with anti-CTLA4 and anti-PD-1. Finally, we demonstrate that KY1055 depletes T_{Regs} and improves the T_{Eff}:T_{Reg} ratio, in vivo, in a CT26 model of tumour growth. KY1055 is progressing through preclinical development for the treatment of solid tumours as a monotherapy and in combination.

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Detection of novel citrullinated targets for cancer therapy

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Stressful conditions in the tumor microenvironment induce autophagy in cancer cells to promote their survival. However, autophagy also causes post-translational modification of proteins, in particular citrullination, which is recognized by the immune system. We have previously shown that killer CD4 T cells which are stimulated by citrullinated vimentin result in strong anti-tumor responses (Brentville et al., 2016). In this study we show that citrullinated vim 415-433, vim 28-49 and enolase 241-260 peptides induce strong CD4 T cell responses that result in anti-tumor immunity against established

tumors. In contrast, the wild type peptides show no anti-tumor responses. This may be related to the fact that many serine proteases cleave after arginine but that citrulline protects from proteolytic digestion. We have shown that wild type vim 415-433, vim 28-49 and enolase 241-260 peptides are all cleaved by trypsin but the citrullinated peptides are protected. However, trypsin is not found within autophagic vesicles so cannot be the enzyme responsible for wild type epitope digestion. In contrast we show the mitochondrial serine protease HTRA2 which is upregulated by ER stress and induces autophagy cleaves wild type but citrullinated vimentin is protected. Realistically *in vivo* several proteases will be involved in the cleavage of these proteins that future studies will identify.
Protein Arginine Deiminases (PADs) are a family of Ca^{2+} dependent enzymes that under cellular stress post-translationally convert arginine to citrulline within protein substrates to generate self-modified neo-antigens. There are many proteins that have been shown to be citrullinated, however, not every arginine residue is converted and not every citrullinated epitope stimulates a T cell response or is presented on MHC-II on tumors. To identify key citrullinated residues we modified *in vitro* vimentin and enolase with both PAD2 and PAD4. Citrullination is confirmed by chemical modification of citrulline with 2,3-butanedione monoxime and antipyrine in a strong acid solution and detection achieved by immunoblotting with an anti-modified citrulline antibody. The citrullinated or wild type proteins are then trypsinized and fragments identified by mass spectroscopy. Interestingly, most of the arginines (>90% PAD2 and around 55% PAD4) in vimentin are citrullinated but only 2/40 stimulate T cell responses in HLA-DR4 mice. The arginine residues in wild type recombinant proteins located within the sequences of interest vim 415-433, vim 28-49 and enolase 241-260 were unmodified in contrast to the deiminated proteins. This study suggests that citrullination may create neo-epitopes due to altered proteolytic digestion which are ideal targets for solid tumors.
Reference: Brentville et al., 2016. Citrullinated vimentin presented on MHC-II in tumor cells is a target for CD4+ T cell-mediated antitumor immunity. Cancer Research 2016 Feb 1;76(3):548-60.

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Identification of receptor binding partners via APEX2 proximity labeling
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Interactions between ligands and receptors play a fundamental role in many biological and pathological processes including the induction of chemotaxis during immune responses. Among the variety of chemokines, there are many, whose receptors still remain unknown. Therefore we aimed to establish a robust and reliable method for identification of receptor binding partners.
By fusing the modified ascorbate peroxidase (APEX2) to the chemokine of interest we are able to selectively biotinylate corresponding receptors. Subsequently these receptors can easily be identified and characterized via western blot, immunofluorescence microscopy or mass spectrometry. Moreover, alterations in binding affinities e.g. due to proteolytic ligand modification can be analyzed by flow cytometry.
In summary, this method enables detection, visualization and quantification of receptor binding, in a faster and more flexible way compared to conventional methods such as co-immunoprecipitation. Use of this method will contribute towards the identification and characterization of immunological signaling pathways.

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Identification and characterization of HLA-Ligands for immunotherapeutic approaches in oropharyngeal squamous-cell carcinoma

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Oropharyngeal squamous-cell carcinoma (OPSCC) arises from the mucosal lining of the oropharynx. In addition to smoking and alcohol, high-risk Human Papillomavirus (HPV) types have been established as the dominant risk factors. Approximately 30-50% of OPSCC are associated with HPV, among which 90% are caused by HPV-16. HPV-associated OPSCC are characterized by the expression of HPV-specific antigens and different mutations in the cancer cell genome. Increasing evidence supports the hypothesis that the repertoire of cancer-associated non-viral antigens also differs between HPV+ and HPV- OPSCC.
In the present study, the HLA ligandome of fresh frozen OPSCC samples will be mapped to identify OPSCC-associated antigens among HPV+ and HPV- patients. To this end, 40 OPSCC samples will be analyzed (20 HPV+, 20 HPV-). The HLA molecules are isolated from tissue samples through cell lysis and immunoaffinity chromatography using antibodies specific for HLA class I and class II molecules. HLA-bound peptides are analyzed by high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS). The HLA-bound peptides are compared to a database containing HLA ligands from various tissues to reveal HLA ligands exclusively presented on OPSCC and not on healthy tissue samples. Whole exome sequencing of RNA and DNA isolated from the respective cancer tissue samples will be performed to complement the database.
So far, HLA ligandome analysis was performed for eight OPSCC. In total, 9726 peptides from more than 5000 source proteins were detected on HLA class I. For HLA class II, 6823 peptides from more than 2000 source proteins were discovered. Within the eight presently analyzed tumor samples HLA class I peptides from 83 proteins were identified of which no peptides have been found on healthy tissue samples so far. For class II, peptides from 179 tumor-exclusive proteins were identified.
The analysis of HLA-ligands can successfully be performed in OPSCC tissue samples. Thus, mass spectrometry and sequencing guided antigen discovery will reveal tumor associated antigens commonly expressed in HPV+ or HPV- OPSCC patients. Immunogenicity testing and bioinformatic integration of the multidimensional datasets will help to select OPSCC-specific antigens for HPV+ and HPV- patients. These antigens may serve as a basis for the development of semi-personalized or personalized vaccines for the treatment of OPSCC.

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T cells against the N345K mutation of PIK3CA

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Somatic point mutations in tumors can generate tumor-specific neoantigens, that may be recognized by T-cell repertoires of individual patients. In search of HLA-A*02:01-restricted, mutated neoantigens in the human HLA-A*02:01$^+$ melanoma model Ma-Mel-66, exome and transcriptome sequencing was

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performed on two melanoma lines (66a and 66b) from distinct skin metastases and on an autologous lymphoblastoid cell line as a germline control. Two hundred and thirty-one expressed somatic non-synonymous point mutations were identified in 66a and 307 in 66b. Ninety-six nonamer (9-mer) and decamer (10-mer) peptides containing mutated sites were predicted to bind to HLA-A*02:01 using the NetMHC4.0 and Immune Epitope Database (IEDB) algorithms. Cutoff values were a binding affinity of ≤500 nM (NetMHC) and a percentile rank of ≤6 (IEDB). A somatic mutation (c.1035T>A; p.N345K) in the functionally relevant C2 domain of PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) was found in both Ma-Mel-66 melanoma lines. PIK3CA mutations occur frequently in cancer and are considered to represent driver mutations. The N345K mutation has been detected in a significant proportion of breast cancers and in other tumor types. Peptides harboring the N-to-K-substitution were predicted to bind to HLA-A*02:01 with high affinity (peptide 338-346: 93.8 nM, percentile rank 1.9; peptide 337-346: 180.7 nM, percentile rank 1.9). MS/MS analyses of K562/HLA-A*02:01 cells stably transfected with cDNA encoding $PIK3CA^{N345K}$ unequivocally demonstrated that both peptides are naturally processed and presented by HLA-A*02:01 although at a low level. While failing to detect $PIK3CA^{N345K}$-specific $CD8^+$ T cells in the peripheral blood lymphocytes of patient Ma-Mel-66, we could generate such T cells by immunizing HLA-A*02:01/K^b transgenic CyA2K^b mice with a synthetic 9-mer $PIK3CA^{N345K}$ peptide comprising residues 338-346. From splenocytes of these mice a monospecific $CD8^+$ T-cell line specific for the peptide was established by weekly *in vitro* stimulation of T cells with T2 cells loaded with the vaccine peptide. More than 90% of these T cells stained with an HLA-A2.1/$PIK3CA^{N345K}$ 338-346 tetramer (obtained through the NIH Tetramer Core Facility). They recognized in ELISPOT assays the mutated PIK3CA 9-mer and 10-mer peptides, but not their wild-type homologues, and also cell lines co-transfected with *HLA-A*02:01*- and *$PIK3CA^{N345K}$*-cDNA after interferon gamma pretreatment. In a next step we will clone their T-cell receptor for further *in vitro* studies. In conclusion, we have demonstrated that $PIK3CA^{N345K}$, encoded by a recurrent driver mutation in cancer, can be targeted by T cells and therefore represents a candidate tumor-specific mutated neoantigen. We will pursue our efforts to generate high-affinity T-cell receptors against $PIK3CA^{N345K}$ restricted by HLA-A*02:01 and other HLA-alleles.

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Human IL-10 (AM0010, Pegilodecakin) in combination with immune checkpoint blockade

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At therapeutic concentrations, PEGylated IL-10 (pegilodecakin, AM0010) stimulates the cytotoxicity, survival and proliferation of intratumoral antigen activated CD8+ T cells in pre-clinical cancer models and in patients. Pegilodecakin activates antigen stimulated CD8 T cells while PD-1 inhibits them, providing a rationale for combining AM0010 with PD-1 inhibitors. A phase 1 clinical trial investigated the tolerability and anti-tumor activity of pegilodecakin alone and in combination with anti-PD1 immune checkpoint inhibitors.
We previously reported partial responses in 4 of 16 RCC pts treated with pegilodecakin monotherapy and 4 of 8 RCC pts (50% ORR) and 2 of 5 of NSCLC pts (ORR 40%) treated with pegilodecakin and pembrolizumab. We enrolled phase 1 cohorts of RCC and NSCLC pts on pegilodecakin + nivolumab as ≥ 2nd line of treatment.
A total of 34 NSCLC pts. were enrolled on pegilodecakin (10-20mg/kg QD, SC) and pembrolizumab

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(2mg/kg, q3wk IV; n=5) or nivolumab (3mg/kg, q2wk IV; n=29). Pts. had a median of 2 prior therapies (range 0-5). 37 RCC patients were enrolled on AM0010 (10-20mg/kg QD, SC) and pembrolizumab (2mg/kg, q3wk IV; n=8) or nivolumab (3mg/kg, q2wk IV; n=29), with a median of 1 prior therapy (1-3) and at least one anti-angiogenic therapy. Tumor responses were assessed by irRC. Serum cytokines, T cell activation (FACS) and peripheral T cell clonality (TCR sequencing) were analyzed.
Pegilodecakin + anti-PD-1 was well tolerated. TrAEs included anemia, thrombocytopenia and fatigue, and were reversible and transient. The incidence of clinically relevant immune related TrAEs was lower than expected from historical controls. As of Oct 29 2017, partial responses (PRs) were observed in 11 of 26 evaluable NSCLC pts (41%), including 4 (of 12) with PD-L1+ < 1% and 4 (of 5) with PD-L1+ >50% cancers. 8 patients with NSCLC had liver metastasis and 6 of 8 had a more than 50% reduction in the liver metastasis.
PRs were observed in 14 of 34 evaluable RCC pts (41%). An additional 15 RCC pts had stable disease (44%), 7 of those had a tumor reduction > 30%. The mPFS and mOS has not been reached, the mFU is 8.9 m (range 0.5-26.5). Updated response data including data on delayed responses and the durability of response will be presented.
The induction of immune cytokines (IL-18) in the serum, invigoration of CD8+ T cells and the increase of newly expanding T cell clones correlated with objective tumor response to pegilodecakin alone and pegilodecakin + anti-PD-1.
Conclusions: AM0010 in combination with anti-PD-1 is well-tolerated in RCC and NSCLC pts, the recommended phase 2 dose is 10ug/kg in combination with an anti-PD-1 agent. The robust efficacy data and the observed CD8 T cell activation is promising and encourages the continued study of AM0010 in combination with nivolumab or pembrolizumab.

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Establishment of a human CD3ε transgenic mouse model to assess anti-tumor efficacy of human T-cell-redirecting bispecific antibodies

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T-cell redirecting therapy has taken a prominent area in immuno-oncology. T cells driven by a tumor-specific antigen, traffic into the tumor and initiate tumor killing. However, this is often hampered by inhibitory factors in the tumor microenvironment. In recent years, researchers have been actively developing T-cell redirecting bispecific antibodies, which binds to a specific tumor associated antigen (TAA) on tumor cells and CD3 (usually epsilon chain, CD3E) on T cells. This physically links the tumor cell to the T-cell which leads to MHC-independent recognition and killing of cells carrying the TAA. Several CD3 recruiting bispecific antibodies that have been approved are now in clinical trials and demonstrate promising efficacy. However, research on new therapeutic T-cell redirecting antibodies is often hampered by a lack of proper *in vivo* models, due to the absence of cross-reactivity with mouse CD3ε. We have now built a human CD3E BAC transgenic model in BALB/c background to address this issue. These transgenic mice express both human- and mouse CD3ε in more than 80% of the T cells. Unlike previously developed transgenic lines, where early T lymphocyte and natural killer cell development were blocked in mice with high copy numbers of the human CD3ε gene, our model is phenotypically normal with levels of T, B , and NK cells comparable to those in wild-type BALB/c mice. In an *ex vivo* T-cell stimulation assay, spleen-derived T cells could be activated by either anti-human CD3 antibody (OKT3) or anti-mouse CD3 antibody (145-2C11), indicated by significantly elevated $CD25^{+}/CD69^{+}$ population, as well as IL-2 and IFNγ release. In an efficacy assay, we inoculated syngeneic mouse CD20-expressing A20 lymphoma cells into the transgenic mice and treated with mCD20xmCD3 or mCD20xhCD3 bispecific antibodies, containing a human- or mouse CD3ε-specific CD3 arm, respectively. We found complete depletion of peripheral B cells 48 hours after dosing of

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either bispecific antibody. Treatment also induced 33% and 39% tumor growth inhibition at day 10, following 3 doses of 1 mg/kg mCD20xhCD3 and mCD20xmCD3 bispecific antibody, respectively. Taken together, our human CD3ε transgenic mice offer a novel model to assess the preclinical *in vivo* efficacy of human CD3-T-cell redirecting therapeutics.

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DSP106 - a novel PD1-CD70 dual signaling protein (DSP) for cancer immunotherapy

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Background: The approval of immunotherapeutic agents working through immune checkpoint inhibition has positively influenced treatment outcomes for cancer patients. However, only a minority of patients achieve durable responses with checkpoint inhibitors. Strategies to improve clinical outcome include combination of different immune checkpoint inhibitors as well as their combination with activation of co-stimulatory receptors on T cells. Here we report the generation of DSP106, a dual signaling protein, comprising the extracellular domain of PD1 fused to the extracellular domain of CD70. DSP106 binds to PD-L1 expressing tumor cells through its PD1 domain and is designed to inhibit PD-1/PD-L1 interaction in the tumor microenvironment thereby removing the inhibitory signal. Simultaneously, DSP106 binding to PD-L1 leads to its surface immobilization, enabling delivery of CD70-mediated CD27 costimulatory signal to tumor localized T cells. This dual immunomodulatory effect of DSP106 is designed to unleash anti-tumor T cell immune responses at the tumor site. Differentiated from antibody combinations or bi-specific antibodies, our DSP platform enables the production of fusion proteins as trimers, a structure that is essential for TNF receptor family activation. The unique DSP composition ensures targeted and increased potency with a better risk/benefit safety profile.
Results: DSP106 was successfully produced in the form of trimers in a mammalian expression system. Both sides of DSP106 bind their cognate counterparts in a Blitz binding kinetic assay and on human tumor and immune cell surface. DSP106 blocks the interaction of PD1 with PD-L1 in an ELISA-based competition assay. In a reporter assay, measuring IL-8 secretion upon binding to CD27, DSP106 activates TNF-R signaling only in the presence of PD-L1 expressing cells. When added to primary human PBMCs, DSP106 augmented the activation of T-cells induced by αCD3 antibody and IL-2 in the presence of PDL-1 expressing cells, resulting in significantly increased secretion of IFNγ. Finally, the *in vivo* activity of DSP106 was demonstrated in a murine P388 leukemia model reducing tumor burden as measured by ascites volume and was superior to a PD1 blocking antibody in a murine CT26 colon cancer model demonstrating tumor growth inhibition.
Conclusions: Based on the data presented here, we demonstrate the feasibility and functional activity of DSP106, a novel DSP fusion protein, providing checkpoint blockade and TNF superfamily co-stimulation in a single molecule. Dual targeting, by the two functional sides of DSP106, offers multiple functionalities that act simultaneously and may result in a synergistic effect. The DSP platform can be designed for selective tumor site or microenvironment targeting and is adaptable to most checkpoint targets.

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Human endogenous retroviruses as a potential reservoir for T cell mediated cancer immunotherapy

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Epigenetic modulation using DNA methyltransferase inhibitors (DNMTi's), such as 5-azacytidine (5-aza-CR) have been shown to affect the cellular immunogenicity in vitro through upregulations of human endogenous retroviruses (HERV) leading to activation of the INFγ response pathway. HERVs comprise up to 8% of the human genome, and may hold a large reservoir of potential tumor antigens. We examined the in vivo efficacy of 5-aza-CR in terms of upregulations of HERV expression during standard treatment regimen, as well as the ability of such HERV transcripts to form T cell antigens leading to measurable T cell recognition upon treatment. We have studied 66 HERV genes that have been shown to be transcribed in human tissues. To identify HERV derived immune recognition, we generated a library of 1169 HERV derived potential antigenic peptides restricted to the 4 most abundant MHC class I molecules in the Caucasian population. Peripheral blood mononuclear cells (PBMCs) from a cohort of 19 patients treated with DNMTi's for different hematological malignancies (MDS, AML, and CMML) were used to detect CD8+ T cells reactive to ERV-derived peptides. We detected CD8+ T cells specific to several HERV-derived peptides both in healthy and diseased individuals. Further, in an additional cohort of patients we examined expression level of these HERVs by RNA seq analysis and compared with healthy individuals demonstrating a disease associated upregulation of HERVs in hematological malignancies.
Presence of T cells reactive to HERV antigens and enhanced expression of HERVs in these malignancies suggest that HERVs may indeed provide a pool of shared tumor associated antigens. These antigens could potentially be enhanced through DNMTi treatment and may provide a target for T cell mediated immunotherapy.

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Depletion of intratumoural regulatory T cells by the anti-ICOS antibody KY1044 in combination with immune checkpoint blockade enhances the anti-tumour response in pre-clinical models.

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The last few years have shown a strong paradigm shift in cancer therapies with the approval of antibodies targeting immune checkpoints. These immune checkpoint blockers (ICBs) are associated with a strong and long-lasting response. However, there is still a high proportion of patients being resistant to ICBs. Although the mechanisms associated with the lack of immune response are multiple, the presence of highly anti-inflammatory regulatory T cells (T_{Regs}) in the tumour microenvironment (TME) is known to negatively affect the response to these therapies. Similarly, high T_{Reg} levels in the TME have been associated with poor prognosis in several cancers. Together, this highlights the

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potential of targeting and depleting T_{Regs} to enhance anti-tumour responses.
The Inducible T-cell costimulator (ICOS/CD278) is induced when T cells get activated. ICOS expression levels vary in different immune cell subtypes and in different tissues. In preclinical mouse tumour models, T_{Regs} ($CD4^+/FOXP3^+$) constitutively express ICOS and the expression of ICOS on T_{Regs} is significantly higher than that on effector T cells (T_{Effs}). In addition, ICOS expression on T_{Regs} is higher in the TME than in the blood or spleen, which makes it a strong candidate for preferential depletion of T_{Regs} in tumours. By immunizing Kymice™ in which the endogenous *Icos* gene has been knocked out, we identified a novel, fully human antibody called KY1044 that bind to ICOS of several species with single digit nM affinity. Using in vitro and in vivo approaches we demonstrate that KY1044 has a dual mechanism of action: (1) it promotes the preferential depletion of intratumoural $ICOS^{high}$ T_{Regs} resulting in an increase in the T_{Eff}:T_{Reg} ratio in the TME; and (2) it stimulates $ICOS^{Low}$ T_{Eff} cells. Using the mouse effector enabled version of KY1044 (mIgG2a) we confirmed a strong anti-tumour efficacy as monotherapy or in combination with surrogates of ICBs. We also demonstrated a tumour antigen specific immunity, as highlighted by the rejection of the original tumour cells in animals cured of the disease and re-challenged by the same cell line. Noteworthy, Pharmacodynamic studies demonstrate long-term depletion of T_{Regs} and a significant increase in the T_{Eff}:T_{Reg} ratio in response to KY1044. In summary, our data demonstrate that targeting ICOS with KY1044 is a valid approach for manipulating the immune system and for inducing a strong anti-tumour response in several indications. The data presented here also warrant the assessment of KY1044 in cancer patients in a clinical trial.

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Glyco-engineering of an anti-CD40 antibody enhances its agonistic activity
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Anti-tumor immune responses are regulated by a number of regulatory and activating receptors. Targeting of the activating receptor CD40 by agonistic IgG1 or IgG2 antibodies is currently tested in clinical trials. Agonistic activity of anti-CD40 antibodies requires receptor multimerization. Anti-CD40 IgG2 antibodies act cross-linking independent, whereas multimerization by anti-CD40 IgG1 is provided by cross-linking Fcγ receptors (FcγR) on immune cells. Glyco-engineering of monoclonal antibodies is a suitable approach to optimize their bioactivity, for example due to modifying FcγR binding. Based on the anti-CD40 antibody ChiLob 7/4 we aimed to generate differently core-fucosylated IgG1 and IgG2 anti-CD40 antibodies using the human expression platform GlycoExpress. The resulting variants were subsequently tested in relevant functional assays.
Glyco-engineering of humanized ChiLob 7/4 IgG1 and IgG2 (hLob) variants results in strongly enhanced affinity to FcγRIIIa as assessed by bead-based AlphaScreen technology. Consequently, glyco-engineered hLob IgG1 shows enhanced antibody-dependent cellular cytotoxicity (ADCC) against CD40 expressing tumor cells. Without crosslinking immune cells only hLob IgG2 variants led to agonistic activity and B cell proliferation representing their FcγR independent effectivity. However, when provided in the context of FcγRIIIa-expressing cells (e.g. NK cells, DCs) comparison of differently Fc-fucosylated hIgG1, but not hIgG2, antibodies showed significantly enhanced agonistic function by the fucose-reduced antibody as measured by an NFκB reporter cell assay. Treatment of *in vitro* generated dendritic cells (DCs) with fucose-reduced hLob IgG1 results in a robust immune response exceeding its high-fucosylated counterpart and both hIgG2 variants as measured by up-regulation of maturation and activation markers on DCs. An allogeneic mixed-lymphocyte reaction (MLR) was employed to show that enhanced DC maturation by fucose-reduced hLob IgG1 translates into improved activation of T cells with a unique activation profile. Besides enhanced immune activation, expression of both, CD40 and FcγRIIIa, on circulating immune cells carries the risk of unwanted side effects. Despite modest cytokine production by the fucose-reduced antibody, as measured by HUVEC and high-density assays, cytokine release was far below critical controls. Furthermore, in autologous ADCC experiments only slight reductions of CD40-high expressing B cells

and DCs could be detected *in vitro*.
Using the GlycoExpress platform we demonstrate that reduction of core fucosylation on the Fc part of an agonistic anti-CD40 antibody results in enhanced FcγRIIIa binding capacity that translated into broader immune activation when used as IgG1 compared to IgG2 antibody without leading to unexpected safety concerns.

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Database matching of de novo sequencing candidates - a new strategy for MHC peptide identification

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Comprehensive mass spectrometric analysis of MHC peptides (immunopeptidomics) is a powerful tool for identifying tumor-specific targets for cancer immunotherapy. However, identification of MHC peptides by classical database search engines, such as Mascot or Andromeda is challenging due to missing protease specificity (non-tryptic peptides) and short peptide length, especially for MHC class I peptides (8-13 amino acids). Thus, searching databases shows limited sensitivity resulting in low identification rates of MHC peptides.
To overcome these limitations, we have developed a new strategy for the identification of MHC peptides that is based on database matching of de novo sequencing candidates (dbNovo). We analyzed a number of publicly available large-scale datasets of MHC class I peptides (systemhcatlas.org) with our new strategy and compared the results with that of the search engines Mascot and Andromeda. On average, dbNovo identified twice as many MHC class I peptides compared to the classical search engines. Motif analysis (Gibbs clustering) of the identified peptides showed that more than 99% of the class I peptides exclusively identified by dbNovo matches to one of the patient-specific MHC peptide motifs. Database matching against tumor-specific protein databases containing tumor-specific SNVs revealed the presence of additional neoantigens in the analyzed samples that could not be identified with the classical search engines.
In addition, de novo sequencing revealed that a substantial part (10 - 25%) of the MHC peptides cannot be identified in protein databases and thus most likely is not derived from classical proteins. Applying dbNovo in combination with a 6-frame translated transcriptome database revealed that many of these unassigned MHC peptides originate from 3'- or 5'-UTRs, from coding regions with non-canonical reading frames, from ncRNAs, or even from antisense RNAs. With our new approach, we identified thousands of these cryptic MHC peptides in the analyzed datasets, by far the largest number of cryptic peptides identified by mass spectrometry so far.
Taken together, our new de novo sequencing-based strategy dbNovo greatly improves the identification rates for MHC class I peptides, enhances the detection of neoantigens, and enables the reliable identification of cryptic peptides.

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HERA-LIGHT, a novel hexavalent HVEM agonist promoting T cell activation and expansion

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The tumor necrosis factor superfamily (TNFSF) member LIGHT (TNFSF14) is known to regulate the activity of a variety of immune cells. Engagement of HVEM (herpes virus entry mediator), one of the

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known receptors of LIGHT, delivers a co-stimulatory signal to support T cell activation and expansion that can help to promote anti-cancer immunity.
The HERA technology platform developed by Apogenix generates fully human hexavalent TNFSF fusion proteins that mimic the natural receptor binding mode in order to co-stimulate T cells. HERA ligands are pure agonists whose signaling capacity does not rely on secondary Fcγ-receptor crosslinking. Here we report the *in vitro* and *in vivo* properties of a novel HERA-LIGHT construct. Similar to all HERA fusion proteins, HERA-LIGHT has been engineered as a perfect molecular mimic of the natural ligand with high clustering capacity for the cognate receptor. The core unit consists of a single chain polypeptide comprising the three minimal LIGHT-subsequences required for folding into a functional trivalent receptor binding domain (RBD). By fusing a silenced IgG1 Fc-domain as a dimerization scaffold to the C-terminus of the RBD we generated HERA-LIGHT, a hexavalent fusion protein.
HERA-LIGHT was expressed in CHO-S cells followed by a lab-scale purification process including AFC- and SEC-based polishing, resulting in homogenous, aggregate-free protein lots. ELISA-based analysis showed that HERA-LIGHT bound both the human and the murine HVEM receptor and revealed good heat- and pH-stress as well as freeze-thaw stability. Analyzing serum samples from a PK study in CD1-mice, the terminal half-life of the compound was 36.5 hours. The short half-life of HERA proteins, relative to antibodies, allows for fast-in/fast-out dynamics essential for combination therapy and mitigates potential side effects associated with immune system overstimulation.
In order to test biological activity, we isolated T cells by magnetic sorting from human PBMCs and stimulated with HERA-LIGHT *in vitro*. Flow cytometric analysis revealed that HERA-LIGHT enhanced activation and proliferation of naïve effector T cells (T_{eff}) following stimulation with anti-CD3 antibody, as determined by CFSE dilution. Importantly, these stimulatory effects were not observed with natural regulatory T cells (nT_{reg}). In contrast, HERA-LIGHT prevented nT_{reg}-mediated suppression and boosted the proliferation of T_{eff} co-cultured with nT_{reg} at various ratios.
In vivo, treatment with a murine surrogate of HERA-LIGHT resulted in significant anti-tumor efficacy in the syngeneic CT26 colorectal cancer model.
In summary, the unique hexavalent design of HERA-LIGHT mediates efficient co-stimulation of T_{eff} even in the presence of T_{reg} cells and independent of secondary crosslinking events. Being true agonists with efficacy independent of T_{reg} depletion, all HERA molecules are unique from current antibody-based concepts rendering them attractive candidates for cancer immunotherapy.

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Theranostic Tumor Imaging of neuroblastoma using radiolabeled αCD276-antibody
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CD276 (B7-H3) is a novel promising target for various solid and hematologic tumors including lung, pancreatic and ovary cancer, melanoma, neuroblastoma, and leukemia. Despite expression on tumor cells, CD276 has been found to be overexpressed in tumor vasculature and to serve as a potential checkpoint inhibitor providing different therapeutic options. Several CD276 targeting therapy approaches including antibodies, radioimmunotherapy, and CAR-T cells are currently under investigation. Antibody-based Theranostic Tumor Imaging using Positron-Emission-Tomography (PET) enables non-invasive whole-body visualization of specific target expression perfectly suitable for therapy stratification, to monitor response to targeted drugs as well as to uncover off-site accumulation. We here report on the preclinical evaluation of a radiolabeled αCD276-monoclonal antibody (mAb) in a neuroblastoma xenograft mouse model.

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The αCD276-mAb used in this study was developed at the University of Tuebingen. For Theranostic Imaging, this antibody was chelator conjugated with DOTAGA at an antibody-to-chelator ratio of 1:15 before radiolabeling with Copper-64 (^{64}Cu). Flow cytometry analyses, radioimmuno and cell western assays were performed to assess target expression and *in vitro* binding characteristics after radiolabeling. 3 weeks before *in vivo* imaging studies, 5 x 10^6 CHP or Jeko-1 control cells were injected subcutaneously in CD1 nude mice. Simultaneous PET/MRI was performed 3 h, 24 h, and 48 h after intravenous injection of 50 µg/12 MBq of ^{64}Cu-DOTAGA-αCD276-mAb. Tumors and organs were analyzed after last imaging time point to assess antibody biodistribution *ex vivo*.
High expression levels of CD276 was found on small cell lung cancer, neuroblastoma, melanoma, prostate cancer, and sarcoma cell lines. Radiochelation yielded high radiochemical purity and radiolabeling efficiency of >90 %. *In vitro* cell binding tests revealed only minimal impairment of the binding affinity and immunoreactivity after radiolabeling. Tumor uptake of the ^{64}Cu-DOTAGA-αCD276-mAb was significantly higher in neuroblastoma bearing mice (7.99 ± 1.33 % injected dose per gram, %ID/g) compared to CD276-negative tumors (3.11 ± 0.61 %ID/g) and control antibody (3.71 ± 0.61 %ID/g), which was confirmed by *ex vivo* gamma counter measurement. No enhanced antibody accumulation was observed in other organs.
Theranostic Imaging will play an increasingly important role in cancer therapy providing essential information for the development and implementation of targeted therapeutics as we move into the era of precision medicine and individualized therapy planning. This study demonstrates excellent potential of the CD276-targeting radioimmunoconjugate. Furthermore, the diverse functions attributed to CD276 offer a wide range investigating the tumor microenvironment. Subsequent *in vivo* studies are currently under investigation to show universal applicability on different solid tumors.

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Expression data integration: advancing immuno-oncology target discovery
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Suppressive immune checkpoint pathways are hijacked by tumors in order to evade the immune system. The immune-evading pathways are currently being clinically targeted, but the therapies are not effective in all patients and all cancer types. More efforts and efficient approaches are needed to identify how responsiveness can be predicted, and to discover novel targets as alternative or combination treatment. In this study, we used GENEVESTIGATOR® for indication prediction and in search of novel targets for cancer immunotherapy. GENEVESTIGATOR® is an analysis tool and database, containing high-quality curated gene expression data from public studies. It allows the user to mine the data of thousands of experiments simultaneously, to identify genes having a very specific profile or indications associated with the transcriptional activity of selected genes.
To predict in which cancers existing immunotherapies might have the strongest effects, the expression of selected immune checkpoint surface molecules across hundreds of tumors and subtypes was investigated. Such an expression meta-profile indicates which cancer types could be suitable for similar immunotherapies. However, the limited number of targets known today, will not cover the need of the variety of cancer patients. Studying genes that are co-regulated with known immune checkpoint targets across immune-oncology studies, provides knowledge of which immunological pathways are affected, as well as novel target candidates. Using GENEVESTIGATOR® for co-regulation studies, several genes were identified as having notably similar regulation patterns as both CTLA4 and PD-1. Some of these are known targets for immunotherapy and confirm the method, while others are novel findings which have not been described previously.
These studies show how GENEVESTIGATOR® can effectively take advantage of the world's high-quality expression data, and help identifying new targets and characterize expression patterns of targets across cancers.

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Setup and validation of a T cell receptor maturation platform resulting in high-affinity binders for engineering of bispecific molecules

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Bispecific T cell receptor (TCR)-antibody fusion proteins against tumor-specific targets represent a promising class of cancer therapeutics. The utilization of a TCR moiety is a major advantage of these molecules as it allows targeting of human leucocyte antigen (HLA)-bound peptides derived from virtually all proteins of the tumor cell regardless of their extracellular or intracellular location. Immatics is developing TCR bispecifics against tumor associated peptide-HLA targets, which have been identified and validated by its proprietary target discovery engine XPRESIDENT®. Immatics has further established a portfolio of technologies to discover and engineer TCRs originating from the natural repertoire of human donors in order to generate soluble and highly potent bispecific T cell receptor therapeutics.
Here we present exemplary results of a platform for affinity maturation of TCRs using yeast display. For maturation we selected a TCR previously validated for its highly specific binding to the XPRESIDENT® peptide-HLA target Ag008-01 with low double digit micromolar affinity. The sequence was converted into a single chain TCR (scTv) comprising the variable domains and a linker sequence and the scTv expression was optimized through the introduction of framework mutations. Yeast libraries with randomized CDR sequences were generated for affinity maturation and scTvs were selected via MACS and multicolor FACS employing increased selection stringency. Mutant scTv candidates from 3 CDR libraries showed improved binding to the target peptide but not to control peptides with high sequence similarity and XPRESIDENT®-confirmed presentation on healthy tissue. When testing different combinations of mutant CDRs we identified scTvs with drastically increased affinity but retained specificity profile.

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Tumour glycan-targeting monoclonal antibodies - Candidate immunogenic cell death inducers

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The tumour glycome is an under-explored source of cancer-specific targets (1). We have generated a panel of glycan-targeting mAbs that have potential for application as cancer therapeutics due to their strong differential tumour versus healthy tissue binding, high functional affinity and their ability to cure mice of metastatic colorectal cancer (2,3). One explanation for the potent anti-tumour responses is the mAbs'ability to induce direct cell death, without the need for immune effector cells or complement. This form of direct cell death, induced by high-level mAb tumour glycan binding, exhibits features of inflammatory/immunogenic cell death (ICD).
Our mIgG3 mAbs bind tumour glycan avidly, provoking increased membrane permeability, cellular aggregation, growth inhibition and leading to significant early-stage release of ATP, preceding late-stage release of the alarmin, high mobility group box 1 protein (HMGB-1). Co-incubation of mAb-treated dying cancer cells with immature monocyte-derived dendritic cells (iDC) instigates iDC maturation and activation, evidenced by increased MHCII, CD80 and CD86 expression. Human hIgG1 formats of our mIgG3 mAbs do not exhibit this form of direct cell killing as they lack the cooperative glycan binding of mIgG3 (4). We have Fc-engineered improved ('i') hIgG1 variants through the selective introduction of mIgG3 residues, thereby recapitulating the mIgG3 direct cancer cell killing. These improved hIgG1 engineered variants show impressive *in vivo* activity in a mouse colorectal

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xenograft model.
mAb-induced ICD, has the potential to synergise with traditional immune effector functions, thus holding tremendous therapeutic promise. Human mAbs with this ability are attractive therapeutic candidates for solid tumour treatment.

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Preclinical development of CD37CAR T-cell therapy for treatment of B-cell non-Hodgkin lymphoma

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T cells modified to express chimeric antigen receptor (CAR) targeting CD19 have produced remarkable clinical responses in patients with relapsed/refractory B-cell acute lymphoblastic leukemia (B-ALL). CD19CAR T-cell therapy has also demonstrated prominent effects in B-NHL patients. However, a subset of patients who relapse after CD19CAR T-cell therapy have outgrowth of CD19-negative tumor cells. Hence, development of alternative CARs targeting other B-cell markers represents an unmet medical need for B-ALL and B-NHL. We confirmed previous data showing that CD37 is widely expressed across multiple subtypes of B-NHL. A second generation CD37CAR was designed from a therapeutically validated anti-CD37 antibody, and its efficacy in T cells was compared to that of CD19CAR. In vitro assessment of cytotoxicity and T-cell function upon co-culture of the CAR T cells with 9 different target B-NHL cell lines demonstrated comparable efficacy between the two CARs when both CD37 and CD19 were expressed. In an aggressive B-NHL xenograft model, CD37CAR T cells were as potent as CD19CAR T cells and significantly prolonged survival. Interestingly, in a second xenograft model, using U2932 lymphoma cells containing a CD19-negative subpopulation, CD37CAR T cells efficiently eradicated tumors and cured the mice while CD19CAR T cells had no effect. We finally showed that CD37CAR recognition was restricted to the B-cell lineage. Thus, CD37CAR T cells effectively eradicate B-NHL tumors, also when CD19 antigen expression is lost. Further development of CD37 CAR therapy in exploratory studies for patients with relapsed and refractory B-NHL is warranted.

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Cloning, expression and functional characterization of an HLA-C*07:01-restricted αβ T-cell receptor against chondroitin sulfate proteoglycan 4 (CSPG4)

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In the context of analyzing the repertoire of anti-tumor T cells in the human melanoma model Ma-Mel-86, melanoma-reactive $CD8^+$ T cells were successfully enriched in autologous mixed lymphocyte-tumor cell cultures (MLTC) by weekly stimulation of peripheral blood lymphocytes with autologous tumor cells. $CD8^+$ T-cell clones derived from MLTC responders were applied to expression screening of cDNA libraries constructed from autologous melanoma cell lines. Two T-cell clones (2c/165 and 11c/73) descending from independent MLTCs were found to recognize chondroitin sulfate proteoglycan 4 (CSPG4) and were directed against the same previously unknown 10mer peptide presented by HLA-C*07:01. Both T-cell clones efficiently lysed autologous tumor cells and strongly cross-reacted with three of four HLA-C*07:01-positive allogeneic melanoma lines. Both clones carried distinct αβ T-cell receptors (TCR) that were identified via the capswitch method and confirmed by PCR-mediated specific amplification of the V regions. The α and β chain cDNAs for both TCR were optimized by replacing the human constant domains with their homologous murine counterparts and cloned in P2A-based bicistronic retroviral constructs. Their expression and functionality were validated by IFNγ-ELISpot assays upon transfer into allogeneic primary T cells. TCR-transduced $CD8^+$ T cells efficiently and specifically recognized melanoma cells and target cells co-transfected with *HLA-C*07:01* and *CSPG4*. In conclusion, we report on the cloning and optimization of two TCR directed against a previously unknown CSPG4 peptide. Due to its relevance for oncogenic pathways and its overexpression in multiple malignancies (with very restricted and low expression in normal adult tissues) CSPG4 has been recognized as a very attractive target for cancer immunotherapy. The high allelic frequency of HLA-C*07:01 (30-40% of Caucasians) supports the therapeutic application of TCR against the CSPG4 epitope identified within this project.

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GARP expressing platelets and the induction of peripheral tolerance: A novel target for anti-tumor-therapy?

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Recently, we were able to show that the soluble form of Glycoprotein A repetitions predominant (sGARP) has strong regulatory and anti-inflammatory properties in vitro and in vivo. Because GARP was first described on platelets, we analyzed the role of GARP expressing platelets in melanoma and its potential as a prognostic marker in more detail.
Platelets are the central cells mediating hemostasis at the site of injury. Furthermore platelets are important modulators of the innate and adaptive immunity through their interaction with immune cells. In case of infection, platelets get activated and are able to modulate the inflammatory process. However, detailed information about the platelet - leukocyte interaction in inflammation is still limited. GARP, first described on platelets and as an activation marker on the surface of activated regulatory T cells (Treg), is known to modulate the bioavailability of TGF-beta and is therefore involved in the

regulation of the peripheral immune responses.
sGARP leads to induction of peripheral Treg as well as to inhibition of tumor antigen-specific $CD8^+$ T cells as shown before.
In the present study, we investigated the effect of platelets on the differentiation and phenotype of $CD4^+$ T cells dependent on GARP. $CD4^+$ T cells where co-cultured with different ratios of platelets and supernatant of activated platelets. Changes in expression of Foxp3, GARP and proliferation on Teff where analyzed via FACS. Additionally, production of effector cytokines IL-2 and IFN-gamma were measured. Presence of sGARP in the supernatant of activated platelets was measured via ELISA. After six days of co-culture, the suppressive capacity of the platelet-conditioned T cells was analyzed with suppression assays.
We were able to detect sGARP in the supernatant of activated platelets. In co-culture, platelets inhibited dose dependently the proliferation and cytokine production of $CD4^+$ T cells, while inducing a strong Foxp3 expression and a suppressive capacity. Using a blocking anti-GARP mAb in the co-culture, we were able to reverse these effects.
In conclusion, our data give evidence that platelets are capable to induce peripheral Treg (pTreg) in a GARP-dependent manner. Through the induction of Treg via GARP expression and sGARP shedding, platelets could be of importance in diseases like cancer where poor prognosis and metastasis are associated with elevated numbers of circulating platelets (thrombocytosis).